THE REDUCTION OF NITRATE, NITRITE AND HYDROXYLAMINE BY *E. COLI*.

by J. LASCELLES AND J. L. STILL

(From the Department of Biochemistry, University of Sydney).

(Accepted for publication 18th April, 1946.)

Stickland (1931) studied the reduction of nitrate to nitrite by washed suspensions of *E. coli* in the presence of lactate and molecular hydrogen. The organism used did not carry the reduction beyond the nitrite stage. Two other strains of *E. coli* were examined by Woods (1938) in the course of a detailed study of the reduction of nitrate by *Cl. welchii*. These two strains were found to reduce nitrate, nitrite and hydroxylamine to ammonia in accordance with the equations:

$$HNO_3 + 4H_2 = NH_3 + 3H_2O \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$
$$HNO_2 + 3H_2 = NH_3 + 2H_2O \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$
$$H_2NOH + H_2 = NH_3 + H_2O \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

Aubel (1938) reported that washed suspensions of *E. coli* reduced nitrate and nitrite to ammonia in the presence of glucose. The same suspensions did not reduce nitrite in the presence of lactate or succinate.

In a study of carrier-linked reactions, Green *et al.* (1934), concluded that the nitrate-reducing enzyme of *E. coli* was linked with either formate dehydrogenase or lactate dehydrogenase via an unknown carrier.

Yamagata (1939) reported the separation, from cells of *B. pyocyaneus*, of an extract containing a flavoprotein having the properties of nitrate reductase.

The present investigation was undertaken to examine the factors concerned in the reduction of these three compounds by *E. coli* in the presence of molecular hydrogen.

METHODS.

Suspensions. These were prepared as described in a previous communication (Lascelles and Still, 1946). In the present work heavier suspensions were required than those used in the study of the oxidation of molecular hydrogen in the presence of methylene blue or oxygen.

Estimation of nitrite. The method of Griess-Ilosvay as described by Stickland (1931) was used.

Estimation of hydroxylamine. The method used was that described by Endres and Kaufmann (1937).

Estimation of ammonia. The method of Conway (1938) was followed.

For all these estimations, the bacterial cells were removed by centrifugation and the estimations carried out on aliquots of the supernatant fluid. In experiments with methylene blue, the dye was removed by absorption on kieselguhr before analysis. Otherwise the procedure adopted was that described in an earlier communication, using Warburg manometers. In all cases the Warburg vessels contained 20 p.c. sodium hydroxide in the centre compartment except when cyanide was present.

REDUCTION OF NITRATE.

The strain of *E. coli* used in this investigation reduced nitrate to ammonia only in the presence of an artificial carrier. It was found most convenient to use benzyl viologen, and the hydrogen uptake per molecule of nitrate was 70 p.c. of that required by calculation for the reduction of the added nitrate to ammonia (Equation 1). Analysis showed that the calculated amount of ammonia was formed.

Without an artificial carrier the nitrate was reduced only to nitrite. In this instance, the hydrogen taken up per molecule of nitrate used corresponded to 70 p.c. of that required by calculation for reduction to nitrite. Analysis showed that the amount of nitrite formed was that required by theory.

The deviation between the actual and the calculated hydrogen uptakes varied with changes of pH and concentration of cells. Blank experiments carried out in an atmosphere of nitrogen showed a considerable amount of reduction, due presumably to the presence of endogenous metabolites which could not be removed completely by washing. This difficulty was encountered also by Stickland (1931).

Fig. 1 indicates the course of the reactions at pH 7·2 and with suspensions of the strength normally used.

A few experiments were carried out with a strain of *A. aerogenes*. In the presence of nitrate this organism took up no hydrogen in the absence of an artificial carrier even though endogenous metabolites did reduce much of the nitrate to nitrite. Addition of boiled suspensions of *E. coli* to such suspensions of *A aerogenes* did not bring about an uptake of hydrogen. Addition of methylene blue resulted in hydrogen uptake to the extent of 70 p.c. of that required by calculation for reduction of nitrate to nitrite. Addition of benzyl viologen in similar circumstances resulted in an uptake of hydrogen equal to 70 p.c. of that required by calculation for reduction of nitrate to ammonia (Equation 1). In both instances analysis showed that the nitrate had been reduced completely to nitrite and ammonia respectively. This same strain of *A. aerogenes* did not show an uptake of hydrogen in the presence of either oxygen or fumarate; but did not do so in the presence of methylene blue.

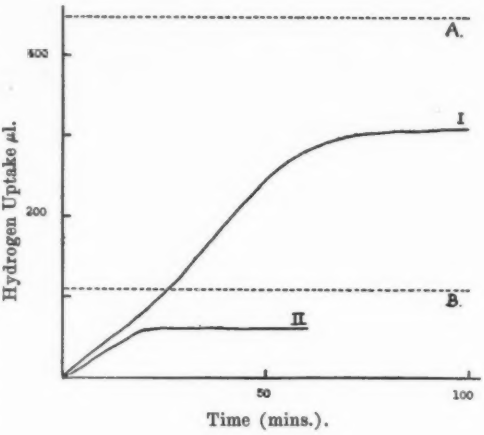


Fig. 1. Course of Hydrogen Uptake in the presence of Nitrate. Each Warburg vessel contained 1·0 ml. suspension, 3·5 mg. dry wt., 1·5 ml. M/5 phosphate buffer at pH 7·2 and distilled water to make a final volume of 3·0 ml. The side arm contained 0·1 ml. M/20 potassium nitrate and 0·1 ml. 0·05 p.c. benzyl viologen or 0·1 ml. distilled water. Curve I. Course of hydrogen uptake in the presence of benzyl viologen. Curve II. Course of hydrogen uptake without added carrier. Line A indicates an uptake of 4 molecules of hydrogen per molecule of nitrate decomposed. Line B indicates an uptake of 1 molecule of hydrogen per molecule of nitrate decomposed.

H-ion Concentration.

Table 1 shows the effect of variation of pH on hydrogen uptake in presence of nitrate.

TABLE 1.

Effect of pH on reduction of nitrate.

Each Warburg vessel contained 1·5 ml. M/5 buffer, 1·0 ml. suspension (3·1 mg. dry wt.) and 0·4 ml. water. The side arm contained 0·1 ml. M/20 KNO_3.

pH.	Hydrogen uptake (µl.). 10 mins.	20 mins.	50 mins.	Percentage of calculated amount (112 µl.).
4·8	2	34	107	95
5·0	8	43	106	95
5·4	16	52	98	88
5·6	32	72	110	98
5·9	36	81	94	84
6·2	29	74	94	84
6·6	21	58	81	72
6·9	25	61	91	81
7·2	21	58	85	76
7·5	27	59	79	71
7·8	20	52	72	64

There was no further hydrogen uptake after 50 minutes at any pH value. There is an initial lag in the hydrogen uptake in the more acid medium as observed by Woods (1938) when studying this phenomenon with suspensions of *Cl. welchii*. That this may be due to a lowered permeability of the cells to nitrate or hydrogen at the lower pH was suggested by the observation that the treatment of the cells with toluene eliminated the lag period. The hydrogen uptake approached the calculated value for complete reduction of nitrate to nitrite at the lower pH values. However, in all cases analyses showed that the nitrate had been completely reduced to nitrite. From the results of blank experiments carried out in an atmosphere of nitrogen, it was clear that the optimum pH for the systems responsible for activation of the endogenous substrates was above pH 6·2.

Concentration of Nitrate.

The effect of variation of the concentration of nitrate on hydrogen uptake is shown in Table 2. The deviation from the calculated uptake at the highest concentration is presumably due to the inhibition by the nitrite produced in the reaction. At least one enzyme concerned would be affected by the nitrite (Lascelles and Still, 1946).

TABLE 2.

Effect of concentration of nitrate.

Each Warburg vessel contained 1·5 ml. M/5 phosphate buffer, at pH 6·0 1·0 ml. suspension (5·0 mg. dry wt.) and 0·4 ml. water. The nitrate was added from the side-arm.

Concentration of nitrate.	Hydrogen uptake (μl.). 10 mins.	20 mins.	40 mins.	60 mins.	70 mins.	Calculated volume H_2.	P.c. of calculated.
M/1,500	25	25	25	25	25	45	56
M/1,050	49	51	51	51	51	64	80
M/600	47	94	94	94	94	112	84
M/300	44	111	194	194	194	224	87
M/150	39	100	200	263	263	448	59

Dilution of Suspensions.

Table 3 shows the effect of dilution of the suspensions on hydrogen uptake in the presence of nitrate.

TABLE 3.

Effect of dilution of suspensions.

Contents of Warburg vessels as above. The side arm contained 0·1 ml. M/20 KNO_3.

Dry wt. of suspension (mg.).	Hydrogen uptake (μl.). 10 mins.	20 mins.	40 mins.
5·0	38	81	81
2·5	21	57	84
1·3	0	14	50
0·7	0	0	0

With dilute suspensions the initial lag was very marked and could not be overcome by the addition of boiled cells or boiled yeast extracts.

Inhibitors.

The reduction of nitrate by molecular hydrogen appeared not to be affected by M/50 hydrazine, M/50 hydroxylamine, M/1,000 2 :4–dinitro–phenol, *m*–, *o*– or *p*– nitro–phenol, 1/10 saturated sodium arsenite. Urethane in M/5 concentration did not inhibit the hydrogen uptake by more than 10 p.c. Incubation of the cells with toluene for 30 minutes at 38° C. appeared to accelerate the hydrogen uptake. This treatment with toluene did, in fact, eliminate the initial lag period and inhibited the endogenous reduction of nitrate. These observations would account for the apparent acceleration of the hydrogen uptake by toluene.

Cyanide in concentrations up to M/1,000 inhibited completely the reduction of nitrate by molecular hydrogen. No differences were detected when cyanide was added to the cells in the presence of hydrogen or oxygen. (See Table 4.)

TABLE 4.

Effect of cyanide.

Contents of the Warburg vessels as before. Suspension 3·4 mg. dry wt. pH 6·2.

Cyanide concentration.	Hydrogen uptake (μl.). 20 mins.	P.c. inhibition.
0	89	—
M/100	0	100
M/500	0	100
M/1,000	9	90

That this inhibition was reversible was shown by the following experiment. Suspension, 3·3 ml., was incubated with 0·33 ml. M/100 KCN in a centrifuge tube for 15 minutes at 38° C. A control with distilled water in place of cyanide was similarly treated. Aliquots of 1 ml. of each were placed in manometer vessels and the remainder of the suspensions were centrifuged. The supernatant was then removed, an equal amount of distilled water added and the cells re-suspended evenly. Aliquots of 1 ml. were again placed in manometer vessels. This procedure was repeated once more. Table 5 summarizes the observations.

TABLE 5.

Reversibility of cyanide inhibition.

Contents of the Warburg vessels as before. Suspension 3·8 mg. dry wt. pH 6·1.

Suspension incubated with	Number of washings.	Hydrogen uptake (μl.). 30 mins.
Water	0	76
KCN	0	11
Water	1	79
KCN	1	77
Water	2	73
KCN	2	78

Sodium azide in concentrations up to M/10,000 almost completely inhibited the uptake of molecular hydrogen in the presence of nitrate. Analyses showed no formation of nitrite in the presence of azide. See Table 6.

TABLE 6.

Inhibition of sodium azide.

Contents of the Warburg vessels as before. Suspenion 3·4 mg. dry wt. pH 6·2.

Concentration of azide.	Hydrogen uptake (μl.). 20 mins.	P.c. inhibition.	Nitrite formed.
0	89	—	0·23 mg.
M/500	0	100	0
M/1,000	0	100	0
M/5,000	8	91	trace
M/10,000	2	98	trace
M/50,000	19	79	0·11 mg.

This inhibition by azide was also shown to be completely reversible by the technique described above for the cyanide inhibition.

Further experiments were carried out using reduced methylene blue as the hydrogen donator. The reduced dye was prepared in a Thunberg tube by reduction with hydrogen in the presence of the cells. The hydrogen was replaced with nitrogen, nitrate then added from the hollow stopper, and the contents analyzed for nitrite after incubation for a given period. Similar experiments without methylene blue were set up. While reoxidation of the leuco-dye could always be observed, reduction of nitrate in the presence of the leuco-dye as determined by analysis for nitrite, could be shown only after addition of inhibitors. In experiments without added inhibitors, the reduction of nitrate was not enhanced by the presence of leuco methylene blue. As mentioned above the cells contained an appreciable quantity of endogenous substrates capable of reducing nitrate. Under these circumstances the reduction of nitrate did not proceed further than the nitrite stage. The results are summarized in Table 7.

TABLE 7.

Effect of inhibitors on reduction of nitrate in the presence of leuco-methylene blue.

Each Thunberg tube contained 1·0 ml. suspension (5·8 mg. dry wt.), 1·5 ml. M/5 phosphate buffer pH 6·2, 0·1 ml. M/27 methylene blue. The inhibitors were incubated with the suspension before addition of nitrate, with the exception of cyanide. The hollow stopper contained 0·1 ml. M/10 KNO_3. Total volume 3·0 ml.

Leuco-methylene blue.	Inhibitor.	P.c. inhibition.
+	Sodium azide M/500	100
−	" " "	100
+	Potassium cyanide M/500	40
−	" " "	89
+	*m*–nitrophenol M/1,000	24
−	" "	56
+	Urethane M/5	24
−	" "	76
+	Toluene saturated	17
−	" "	36

From these results it seemed that in some instances, the inhibitors had a greater effect on endogenous systems than on the nitratase. In these experiments also the inhibition by azide was shown to be reversible by washing the cells with distilled water. Altogether it would seem that azide is the most effective inhibitor of nitrate reduction.

REDUCTION OF NITRITE.

In the absence of an artificial carrier, this strain of *E. coli* did not reduce nitrite. Methylene blue was not effective. In the presence of benzyl viologen and the optimum concentration of nitrite, the hydrogen uptake corresponded with that required by calculation for complete reduction of the nitrite to ammonia (Equation 2). Analysis showed that the added nitrite was completely reduced to ammonia. In an atmosphere of nitrogen, there was only a very small reduction of nitrate due to endogenous substrates, under these conditions.

H-ion Concentration.

When nitrite was used as acceptor difficulty was met in determining the effect of variation of pH, as at least one of the enzymes concerned was more sensitive to nitrite at the lower pH values. This was overcome to some extent by using a low concentration of nitrite. Tables 8 and 9 summarize these observations.

TABLE 8.

Effect of pH on reduction of nitrite.

Each Warburg vessel contained 1·0 ml. suspension (3·5 mg. dry wt) 1·5 ml. M/5 buffer, and 0·3 ml. water in the main compartment. The side arm contained 0·1 ml. 0·05 p.c. benzyl viologen and 0·1 ml. M/40 $NaNO_2$.

Buffer.		Hydrogen uptake (μl.). 40 mins.
Phosphate pH	6·7	41
"	6·9	31
"	7·2	43
"	7·5	50
"	7·8	166
"	8·0	153
Borate	8·5	151
"	9·0	124
"	9·6	81

Concentration of Nitrite.

This was studied at three different pH values as shown in Table 8.

TABLE 9.

Effect of concentration of nitrite.

Each Warburg vessel contained 1·5 ml. M/5 phosphate buffer, 1·0 ml. suspension (2·4 mg. dry wt.), 0·3 ml. distilled water. In the side arm, 0·1 ml sodium nitrite and 0·1 ml. 0·05 p.c. benzyl viologen.

Concentration of nitrite.	pH.	10 mins.	20 mins.	40 mins.	Calculated uptake (μl.).
M/2,400	6·9	74	74	74	84
M/1,200	6·9	33	43	51	168
M/600	6·9	13	13	21	336
M/300	6·9	3	5	19	672
M/2,400	7·2	73	78	78	84
M/1,200	7·2	103	164	170	168
M/600	7·2	25	33	38	336
M/300	7·2	8	11	11	672
M/2,400	7·6	71	78	78	84
M/1,200	7·6	96	145	145	168
M/600	7·6	49	75	89	336
M/300	7·6	24	38	38	672

Effect of Concentration of Cells.

Dilution of the suspension beyond 0·6 mg. dry wt./ml. resulted in a sharp decline in the rate of hydrogen uptake. This was not modified by the addition of boiled suspensions of the organism.

Inhibitors.

The effects of a number of enzyme inhibitors are summarized in Table 10. With the exception of toluene the inhibitors were incubated with the suspension during the equilibration period. Treatment with toluene was the same as described above.

TABLE 10.

Effect of inhibitors.

Contents of Warburg vessels as above. Nitrite 0·1 ml. M/40. Suspension dry wt. 4·0 mg. pH 7·6.

Inhibitor.	Concentration.	P.c. inhibition.
KCN	M/500	95
NaN_3	M/1,000	85
Toluene	satd.	88
2: 4–DNP	M/1,000	99
m–nitrophenol	M/1,000	99
o–nitrophenol	M/1,000	87
p–nitrophenol	M/1,000	87
Hydrazine	M/50	0
Sodium fluoride	M/50	13
,, arsenite	1/10 satd.	55
Urethane	M/5	22

The outstanding differences between the nitrate-reducing system and the nitrite-reducing system were in respect of the inhibition due to toluene and the nitrophenols.

REDUCTION OF HYDROXYLAMINE.

This was studied because hydroxylamine is a possible intermediate in the reduction of nitrite to ammonia, even though attempts to demonstrate its formation have not been successful (Woods, 1938).

Uptake of hydrogen in the presence of hydroxylamine did not occur without the addition of an artificial carrier. Methylene blue was not effective, but benzyl viologen was satisfactory. In the presence of benzyl viologen the hydrogen uptake corresponded with that required by calculation to reduce the hydroxylamine to ammonia according to Equation 3. Analysis showed that the hydroxylamine was quantitively reduced to ammonia. In an atmosphere of nitrogen there was a very small blank reduction of hydroxylamine.

H-ion Concentration.

The variation of hydrogen uptake with variation in pH is shown in Table 11.

TABLE 11.

Effect of pH on reduction of hydroxylamine.

Each Warburg vessel contained 1·0 ml. suspension (4·1 mg. dry wt.) 1·5 ml. buffer M/5, 0·3 ml. water. The side arm contained 0·1 ml. 0·5 p.c. benzyl viologen and 0·1 ml. M/20 hydroxylamine.

Buffer.	pH.	Hydrogen uptake (μl.). 10 mins.
Phosphate	7·2	17
"	7·6	53
"	8·0	81
Borate	8·5	74
"	9·0	89
"	9·9	85

Woods (1938) working on the similar reaction in *Cl. welchii* observed that the optimum pH of this reaction was above 8·5.

Concentration of Hydroxylamine.

There is an optimum concentration of hydroxylamine at about M/150 as shown in Table 12. Actually it had been found previously that M/50 hydroxylamine completely inhibited the reduction of methylene blue by molecular hydrogen in the presence of much more dilute suspensions of the organisms.

TABLE 12.

Effect of concentration of hydroxylamine.

Contents of Warburg vessels as before. Suspension 4·6 mg. dry wt. pH 8·0.

Concentration of hydroxylamine.	Hydrogen uptake (μl.). 10 mins.
M/1,200	33
M/600	87
M/300	154
M/150	242
M/75	110
M/50	94

Effect of Dilution.

As with nitrite, dilution of the suspension resulted in a sharp fall in the rate of hydrogen uptake in the presence of hydroxylamine. This became very marked in suspensions below 0·6 mg./ml. dry wt. Addition of boiled suspensions of the organism did not restore the activity.

Inhibitors.

The effects of a number of inhibitors are summarized in Table 13.

TABLE 13.

Effect of inhibitors on reduction of hydroxylamine.

Contents of Warburg vessels as before except that 0·1 ml. M/5 hydroxylamine was added from the side arm. pH 8·0. Suspension during the equilibration period, with the exception of toluene, which was used as described above.

Inhibitor.	Concentration.	Pc. inhibition.
KCN	M/50	99
Sodium azide	M/1,000	84
Toluene	satd.	80
2: 4–DNP	M/1,000	83
m–NP	M/1,000	85
Hydrazine	M/50	62
Sodium fluoride	M/50	69
" arsenite	1/10 satd.	80

Thus with the exception of fluoride and hydrazine the effects of inhibitors are much the same as in the reduction of nitrite. The differential effect of fluoride on the two reductions was used in an unsuccessful attempt to demonstrate hydroxylamine as an intermediate in the reduction of nitrite to ammonia.

DISCUSSION.

The action of *E. coli* on nitrate seems to vary among strains of that organism. There are differences in both the extent of reduction and the rate. Thus the organism used by Stickland (1931) and that used in this investigation do not normally carry this reduction beyond the nitrite stage. The two strains used by Woods (1938) carried the reduction completely to ammonia without the addition of carriers, but at different rates. It would seem that the extent of reduction beyond the nitrite stage depends upon the presence of a carrier or carriers in the organism. These were present in the strains used by Woods, but not in those used by Stickland or the authors. It is not possible to decide whether the organism used by Stickland lacked the enzymes activating nitrite and hydroxylamine, or the carriers required, or both.

The nature of such carriers is not known, but the work of Yamagata (1939) on *B. pyocyaneus* suggests that a flavin compound may be involved in such coupling. Some of the differences between the systems which may be coupled with hydrogenase in this strain of *E. coli* are summarized in Table 14.

TABLE 14.

Effect of inhibitors on oxidation of molecular hydrogen.

Acceptor.	Inhibitor.					
	KCN.	CO.	NaN_3.	2:4–DNP.	NH_2OH.	Toluene.
Methylene blue	+++	+++	+	(++)	+++	0
Oxygen	+++	−	Acceln.	(+++)	+	+++
Nitrate	+++	−	+++	0	0	0
Nitrite	+++	−	+++	+++	−	+++
Hydroxylamine	+++	−	+++	+++	−	+++

+ = inhibition, 0 = no inhibition, ( ) = after shaking the suspension in oxygen.

The evidence supports the hypothesis that different enzymes are responsible for the reduction of nitrate on the one hand, and nitrite and hydroxylamine on the other. Actually, azide affected reduction of nitrate at much lower concentrations than it did the reduction of nitrite and hydroxylamine. The reversible inhibition of nitratase by azide recalls the similar effect of this substance on catalase, an iron protoporphyrin enzyme (Blaschko 1935; Keilin and Hartree 1936).

In some experiments in which nitrate was reduced to ammonia, attempts were made to isolate hydroxylamine as an intermediate compound. These attempts were no more successful than those of Woods (1938). No attempt was made to devise experiments similar to those of Aubel (1938), in which hyponitrite was an intermediate in the reduction of nitrate to ammonia.

Aubel *et al.* (1937, a, b), also made the point that in experiments in which reduced dyes were incubated in the presence of nitrite, the dyes were re-oxidized independently of enzymes. This suggested that in the present experiments where artificial carriers were used, reduction beyond the nitrite stage was purely non-enzymic as far as re-oxidation of the dye was concerned. However, the fact that treatment of the cells with toluene prevented further reduction of nitrate beyond the nitrite stage, in the presence of benzyl viologen, seemed to preclude this possibility. Also, according to Aubel *et al.* (1937, b), in experiments in which leuco methylene blue was used as the hydrogen donator, re-oxidation of the dye would have been due to the non-enzymic action of nitrite produced by the endogenous

donators in the suspensions. Reference to Table 7 does not support this hypothesis. Thus treatment of the suspensions with some inhibitors reduced appreciably the endogenous nitrite production, whereas in the presence of leuco methylene blue a greater production of nitrite occurred. This suggested that the leuco dye donated hydrogen to nitrate. The systems responsible for the activation of the endogenous substrates were apparently more sensitive to these inhibitors than nitratase.

SUMMARY.

The reduction of added nitrate, nitrite and hydroxylamine by washed suspensions of *E. coli* in the presence of molecular hydrogen has been studied.

The reduction of nitrate beyond the nitrite stage required the addition of a carrier. A study of the effects of inhibitors supported the view that the systems responsible for the reduction of nitrite and hydroxylamine are distinct from nitratase.

REFERENCES.

Aubel, E. (1938): C.R. Soc. Biol. Paris, 128, p. 45.
Aubel, E., Schwarzkopf, O. and Glaser, E. (1937): (a) *Ibid.*, 125, p. 12; (b) *Ibid.*, 125, p. 223.
Blaschko, H. (1935): Biochem. J., 29, p. 2303.
Conway, E. (1938): Micro-Diffusion Analysis. D. van Nostrand Co., New York.
Endres, G. and Kaufmann, L. (1937): Liebigs Ann. 530, p. 184.
Green, D. E., Stickland, L. H. and Tarr, H. A. L. (1934): Biochem. J., 28, p. 1812.
Keilin, D. and Hartree, E. F. (1936): Proc. Roy. Soc. Lond. B. 121, p. 173.
Lascelles, J. and Still, J. L. (1946): Austral. J. exp. Biol., 24, p. 37.
Stickland, L. H. (1931): Biochem. J., 25, p. 1543.
Woods, D. D. (1938): *Ibid.*, 32, p. 2000.
Yamagata, S. (1939): Acta Phytochim., 11, p. 145.

ADDENDUM.

Note added during proof. August 6, 1946.

Recently in this laboratory, Back *et al.* (1946), have obtained cell-free extracts of *E. coli* which catalyse the reduction of nitrate, nitrite, hydroxylamine, fumarate, methylene blue and molecular oxygen by molecular hydrogen. The extracts were prepared using the ground glass technique of Kalnitsky *et. al* (1945).

Back, K. J. C., Lascelles, J. and Still, J. L. (1946): Austral. J. Sci., 9 (in press).
Kalnitsky, G., Utter, M. F. and Werkman, C. H. (1945): J. Bact. 49, p. 595.

ANTIBACTERIAL ACTIVITY IN MEMBERS OF THE HIGHER FUNGI

1. *CORTINARIUS ROTUNDISPORUS*[1] AND *PSALLIOTA XANTHODERMA* GENEV.

by NANCY ATKINSON

(From the Institute of Medical and Veterinary Science, Adelaide).

(Accepted for publication 22nd April, 1946.)

Antibacterial activity appeared in watery extracts of specimens of *Cortinarius rotundisporus* and *Psalliota xanthoderma* collected in various parts of South Australia. The activity was readily demonstrated by the cylinder-plate test of Heatley (1944) and affected not only the Gram-positive coccus *Staph. aureus*, but also the Gram-negative bacillus *Bact. typhosum* and the acid-fast types, *Myco. phlei* and the vole type of tubercle bacillus. On account of this wide range of activity the antibacterial substances of these fungi were considered worthy of further investigation and characterization. According to the results so far obtained the substance responsible for the activity of *Cortinarius rotundisporus* is probably different from that producing activity in *Psalliota xanthoderma*. Thus, in crude extracts of *Psalliota*, the activity was greater against *Bact. typhosum* than against *Staph. aureus;* similar extracts of *Cortinarius* possessed markedly greater activity against *Staph. aureus* than against *Bact. typhosum*. Also the activity of the *Cortinarius* extract as compared with that of the *Psalliota* extract was more stable to heat. The possibility remains, however, that more than one substance may be responsible for the activities of these extracts. So far, no highly purified material has been prepared, but a preliminary account of some of the characteristics of crude and partially purified preparations appears in the following report.

MATERIALS AND METHODS.

The Fungi.

All specimens of *Cortinarius rotundisporus* were gathered in the hills around Adelaide. This toadstool is not a common type and usually comes up only in late winter and spring. Consequently the total amount procured was small, about one kilogramme, and there is little likelihood of finding more of it before the end of next winter. The extent of the investigations was therefore rather limited for lack of material. A concerted effort next season should result in the gathering of a much larger quantity with which to continue and expand these investigations.

Specimens of the edible mushroom, *Psalliota xanthoderma*, were gathered from country districts widely separated throughout South Australia and from city parks and suburban gardens in Adelaide. Through the summer this mushroom came up regularly after heavy rain.

Although all the specimens of *Cortinarius* used here were identified as *rotundisporus*, small variations occurred among them; each variety showed, however, the characteristic antibacterial activity. Slight variations also appeared among the specimens of *Psalliota* identified as *xanthoderma*, but all showed an iodine brown stain when cut or scraped, and all showed the typical antibacterial activity. Professor J. B. Cleland (1942) describes these two fungi, and I am greatly indebted to him for identifying all the fungi dealt with here.

Tests for Antibacterial Activity.

All qualitative tests were made by the cylinder-plate method of Heatley (1944), using *Staph. aureus* (standard culture for penicillin assay) in one set of plates and *Bact. typhosum* in another. The degree of activity was roughly gauged by the size of the zone of inhibition. Unless otherwise stated the medium employed was nutrient agar.

---

[1] *C. rotundisporus* Clel. et Cheel was described from New South Wales specimens. The South Australian specimens tested, at present referred to this species, differ slightly from the type and may eventually require varietal or specific separation. J.B.C.

Antibacterial titres were determined by serial dilution of the material in nutrient broth or other suitable culture medium which was then inoculated with a standard amount of the test organism and incubated; the antibacterial titre was the highest dilution completely inhibiting the growth of the test organism under set conditions.

RESULTS.

Antibacterial Activity of Crude Watery Extracts.

The cap and stem of the fungus were both active. Extracts were prepared by grinding up the whole fungus with a little water in a mortar, squeezing out the liquid through muslin and then filtering through paper. At least six such extractions were required to get out the greater part of the activity. Some typical results showing the approximate sizes of the zones of inhibition are as follows:

	Vole type tubercle bacillus.	*Myco. phlei.*	*Staph. aureus.*	*Bact. typhosum.*
Cortinarius rotundisporus	Not done	20 mm.	20 mm.	10 mm.
Psalliota xanthoderma	22 mm.	22 mm.	25 mm.	27 mm.

The zones of inhibition with *Myco. phlei* and the vole bacillus were measured after 48 hours' incubation at 37° C. and were not therefore comparable with those for *Staph. aureus* or *Bact. typhosum*. In the tests with these two organisms a marked difference appeared between the two extracts. The *Cortinarius* extract was more active against the staphylococcus than against the typhoid bacillus; the *Psalliota* extract showed the reverse effect; on the two acid-fast types this extract had a similar effect. For providing the culture of the vole bacillus I wish to thank Dr. F. G. Morgan.

Activity against various other bacteria was detected by stroking a portion from each culture in a single line radiating from a cylinder placed in the centre of a plate of nutrient agar. The cylinder had previously been filled with extract and the plate left in the cold overnight to allow good diffusion of the active substance into the agar round the cylinder. On incubation inhibition of growth of sensitive cultures occurred in the part of the inoculum close to the cylinder. The *Cortinarius* extract produced good inhibition of *Bact. coli, B. subtilis, C. diphtheriae mitis, N. catarrhalis* and a Group A streptococcus; the *Psalliota* extract strongly inhibited the growth of *Bact. coli, B. subtilis* and a Group B streptococcus. Neither extract had any effect upon *Ps. pyocyanea*. The *Psalliota* extract inhibited to much the same extent the growth of both a penicillin-sensitive and a penicillin-insensitive staphylococcus.

Stability of the Antibacterial Activity.

(a) *Effect of acidity.* Both the *Cortinarius* and the *Psalliota* crude extracts were naturally acid, about pH 4, and both retained activity when stored at natural pH for at least two months at 2° C.

A portion of each extract was adjusted with dilute HCl to pH 2, left for 20 minutes at room temperature and then adjusted with dilute NaOH to pH 7 and tested for activity against *Staph. aureus* and *Bact. typhosum*. No obvious alteration had occurred in the activity of either extract as compared with that of the control which consisted of another portion of the same extract adjusted to pH 7. Thus, in crude extracts, the antibacterial substances were not readily destroyed by acid.

(b) *Effect of alkalinity.* A portion of each extract was adjusted to pH 9, left at room temperature for 20 minutes and then adjusted to pH 7 and tested for activity as above. No obvious inactivation occurred in either extract, nor in some *Psalliota* extract kept at pH 8 for 1 week at 2° C. Evidently the antibacterial substances in the crude extracts are not highly susceptible to alkali.

(c) *Effect of heat.* A portion of each extract, adjusted to pH 7, and another portion unadjusted were placed in boiling water and tested for activity after 5 and 15 minutes. In the unadjusted *Psalliota* extract a precipitate appeared. No obvious alteration occurred in the activity of the *Cortinarius* extract after 15 minutes at 100° C. The activity of the *Psalliota* extract quickly disappeared and in the unadjusted portion after five minutes' heating was only faintly evident, producing a zone of inhibition of 9 mm. with *Bact. typhosum* and no inhibition of *Staph. aureus*. In the portion at pH 7, destruction was not so great, as the residual activity, after five minutes' heating, was manifested by inhibition of *Bact. typhosum* in a zone of 12 mm., but *Staph. aureus* was unaffected; no activity remained after 15 minutes at 100° C.

Another marked difference between the two extracts, namely the survival of the activity of the *Cortinarius* extract and the complete destruction of the activity of the *Psalliota* extract after 15 minutes at 100° C. is thus demonstrated.

(d) *Effect of storage of the fungi.* Not enough specimens of *Cortinarius* were available for extensive investigations of survival of activity with keeping, but toadstols kept at 2° C. for 3–4 days still yielded active extracts. Specimens of *Psalliota* maintained their activity well after gathering, and even when in a state of advanced decay many provided active extracts,

numerous batches of these mushrooms travelled by rail or post to the laboratory, taking a few days in transit; very few had lost their activity when they arrived, and many were kept for two weeks at 2° C. and were still highly active.

(e) *Effect of adsorbent agents.* There was insufficient *Cortinarius* extract for the performance of these tests. The *Psalliota* extract was shaken with varying amounts of activated charcoal (Schering) with the result that, except with the smallest amount, activity disappeared from the extract and did not appear on the carbon. Even with the smallest amount (approximately 10 mg. charcoal in 40 ml. of extract) the activity of the extract, after filtering off the charcoal, was reduced so that it produced a zone of inhibition of 11 mm. with *Staph. aureus* and 16 mm. with *Bact. typhosum* as compared with 18 mm. and 21 mm. respectively for the control. The lost activity did not appear on the charcoal which showed only a faint inhibition of *Bact. typhosum*. Apparently the charcoal destroyed the active substance.

Filtration through a Seitz EK pad caused a small drop in activity of crude watery extracts of *Psalliota*. Impure extracts of *Psalliota* in ethyl acetate lost most of their activity on passage through a short column of Brockman's alumina and no activity was detectable in the alumina. But when commercial alumina (washed, treated with HCl and dried) was substituted for Brockman's alumina some of the activity went through in the percolate and some was adsorbed by the alumina. The active substance was easily eluted from the alumina with water.

Thus, the activity of *Psalliota* extracts was readily inactivated by the strong adsorbents and not readily adsorbed by the weak adsorbents.

(f) *Effect of drying* in vacuo *from the frozen state.* Portions of the crude extracts were frozen solid and dried *in vacuo* without thawing or reaching a temperature higher than 30° C. The *Cortinarius* extract showed no diminution of activity after this treatment; the *Psalliota* extract lost a small part of its activity. Destruction of some of the activity of the *Psalliota* extract under these gentle conditions of drying emphasized the instability of the antibacterial substance. Its easy destruction by certain agents had been demonstrated already in the tests on heat-resistance and effect of adsorbents. Compared with the activity of *Cortinarius* that of *Psalliota* was much more labile.

Extraction and Purification of the Antibacterial Substances.

So far no highly purified preparation has been obtained from either fungus, but several methods have been explored and have revealed further characteristics of the antibacterial substances. The small amount of *Cortinarius* available allowed only limited investigation. The antibacterial substance was extracted from the ground-up toadstools into distilled water by thorough mixing and then squeezing through muslin. Four such extractions, using about 30 ml. of water for each, produced almost equally active solutions which were pooled and used for the subsequent experiments. Attempts were made to extract the active substance into ether or chloroform at pH 10, pH 2 and pH 7. Much the same amount of activity (representing only a portion of the total) passed into the solvent at all pH values; in the extraction, chloroform appeared to be a little more effective than ether. The whole of the remaining watery extract, without adjustment of pH, was thoroughly shaken four times with an equal volume of chloroform. The pooled chloroform extracts were dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo* to a small volume and left at 2° C. in a desiccator which was evacuated each day until all solvent had disappeared. The resultant dry residue was very small, approximately 5 mg., and consisted of apparently colourless, finely crystalline material accompanied by some dark purple amorphous material. The whole of this residue was dissolved in 2 ml. of water and produced a deep purple solution in which the approximate concentration of toadstool material was 1 in 400. So little solution was available that accurate titration of its antibacterial activity was impossible, but rough tests indicated approximately antibacterial titres for the solid material of 1/300,000 for *Staph. aureus*, 1/10,000 for *Bact. typhosum* and 1/80,000 for *Myco. phlei*. The investigation will be continued when more *Cortinarius* is available.

The antibacterial substance of *Psalliota* was easily extracted by grinding the mushrooms with a pestle and mortar after adding a small volume of water, just enough thoroughly to wet them. The extract was obtained by squeezing the ground-up mushrooms in muslin. Active extracts resulted from twelve such extractions on the same mushroom pulp. The activity fell off somewhat in the later extractions; in the earlier extractions typical results for the zones of inhibition of *Staph. aureus* and *Bact. typhosum* were 25 mm. and 27 mm. respectively. Some, but by no means all of the antibacterial activity was extracted by ethyl acetate which was added to the ground-up mushroom. This extract was dark yellow and, on shaking with water, gave up part of its colour and antibacterial activity to the watery layer. Thus a partial purification was effected but the losses occurring during the process made it too wasteful to use with the limited supply of mushrooms available. The same objection applied to the method of partial purification of dry ethyl acetate extracts by passing them through an alumina column. As already mentioned, some activity went through and some remained in the column, in which four bands appeared: the topmost was small and dark brown, the next was larger and pale brown, and was followed by a broad yellow band, and finally a pale pink band. The bands were separated and eluted first with ether and then with ethyl acetate neither of which removed much activity; they were finally

eluted with water in which good activity appeared from the dark brown, pale brown and yellow bands; no activity was found in the pink band. The ethyl acetate percolate was allowed slowly to evaporate in the cold; colourless, needle-like crystals of inactive material separated and the activity of the solution gradually disappeared.

A portion of the material obtained from drying some frozen crude watery extract *in vacuo* was thoroughly extracted with ether which remained practically colourless; the ether was removed *in vacuo*, and the residue taken up in water. The colourless solution showed the greatest activity so far obtained, namely, inhibition zones of 32 mm. with *Staph. aureus* and 37 mm. with *Bact. typhosum*. This method will be further exploited when more mushrooms are available.

Effect of Serum and Various other Substances on the Activity of the Extracts.

Ox serum was mixed with crude, watery extracts of *Psalliota* and *Cortinarius*, giving a final dilution of 1 in 4; after standing at room temperature for two hours the mixtures were tested and produced zones of inhibition of much the same size as those produced by the control mixture in which water had been substituted for serum. Apparently serum under these conditions does not affect the activity of either extract.

The crude watery extracts were mixed with thioglycollic acid giving a final concentration of approximately M/200. After standing for two hours at room temperature, the mixtures were tested and the size of the zones of inhibition produced by the *Psalliota* extract were smaller than those of the control; the *Cortinarius* extract seemed unaffected.

The effect of glycerine on the activity was investigated by using 5 p.c. glycerine agar in the cylinder-plate test and comparing the result with that on ordinary nutrient agar. With *Psalliota* extract no significant difference in the size of the zones of inhibition appeared with *Staph. aureus*, *Bact. typhosum* or *Myco. phlei*.

The Toxicity of the Extracts.

Of the final partially purified solution obtained from the *Cortinarius*, 0·25 ml. was injected intramuscularly into the hind leg of a mouse. No general reaction occurred, but the injected leg swelled and the mouse refused to use it for about 24 hours. By this time the swelling had subsided and the mouse commenced to use the leg again. Aftr 48 hours the mouse appeared normal. This slight toxic effect may have been due to impurities still present in the extract. Owing to scarcity of material no further tests in animals could be made.

A crude extract of *Psalliota* giving an inhibition zone of 27 mm. with *Bact. typhosum* was tested in mice. In one mouse three intraperitoneal injections of 0·25 ml. given three-hourly had no apparent effect; 0·5 ml. fed to a mouse three times a day at intervals of three hours for three days did not affect the animal; three intravenous injections of 0·2 ml. given three-hourly also had no effect. The antibacterial substance appears therefore not to be highly toxic; but a more convincing demonstration of its low toxicity awaits the preparation of a more concentrated, more highly purified extract.

DISCUSSION.

Over 200 types of toadstools and mushrooms have been investigated in this laboratory for antibacterial activity, but only five showed any activity. Of these, three (all species of *Cortinarius*) were active against *Staph. aureus* but not against *Bact. typhosum;* the other two, namely *Cortinarius rotundisporus* and *Psalliota xanthoderma,* were active against both these bacterial types. Further investigations revealed activity also against the acid-fast type *Myco. phlei;* the *Psalliota* extract was also tested and found active against the vole tubercle bacillus. This result suggested that antibacterial activity against other *Mycobacteria,* notably the human and bovine types of tubercle bacillus, may also be manifested. Tests for action on these organisms have not yet been performed owing to lack of material.

The locality from which the *Psalliota* specimens were gathered did not affect their activity, as specimens from districts a hundred or more miles from Adelaide were just as active as those gathered in suburban gardens. Apart from their antibacterial activity the main distinguishing character of these mushrooms was the production of an iodine brown stain on any surface immediately after rubbing or cutting.

Both fungi provided antibacterial extracts with a wide range of activity, affecting not only those bacteria mentioned above, but also *Bact. coli., B. subtilis* and various streptococci. The *Psalliota* extract was active against a staphylococcus relatively insensitive to penicillin; this fact and its activity against other bacteria insensitive to penicillin indicate that the mode of action of the antibacterial substance is different from that of penicillin and investigations on this aspect are

proceeding. These results also suggest that the *Psalliota* substance may be useful in fields outside the reach of penicillin therapy.

The suppression of activity of the *Psalliota* extract by thioglycollic acid was slow; on the other hand, this acid had little effect upon the *Cortinarius* extract. The nature of the test ensured that the antibacterial substance was in contact with the SH compound in the cylinder for the whole of the test—whether they both diffused out relatively equally into the agar was not established, but through at least part of the zone of inhibition both substances would probably be present. The SH compound was therefore not an active suppressor of the antibacterial action of *Psalliota* or *Cortinarius*.

The lack of suppression of the activity of either *Psalliota* or *Cortinarius* extract by serum bodes well for the possibility of their acting *in vivo* satisfactorily. Another point in favour of the possibility of these antibacterial substances acting as chemotherapeutic agents is the apparent lack of toxicity of crude *Psalliota* extracts and the not very marked toxicity of the partly purified *Cortinarius* extract. The *Psalliota* substance seems the more interesting of the two, as the crude extracts have a relatively similar effect upon *Bact. typhosum, Staph. aureus, Myco. phlei* and the vole bacillus, suggesting that the substance when purified may have a wide range of application in much the same dose. Attempts to purify the substance have resulted so far in the production of a brown oil. The isolation is complicated by the lability of the material, the activity of which is destroyed by moderate heat, adsorbents or simply by keeping in watery solution for some weeks in the cold. By drying the watery extracts from the frozen state the material may be preserved in active form for some time. The extracts of the next batch of mushrooms will be so treated, and the dry residues extracted with various organic solvents in a further attempt to obtain a purified compound.

Further work on *Cortinarius rotundisporus* must await new supplies next season.

SUMMARY.

Antibacterial activity against a wide range of bacteria, including acid-fast types, was found in watery extracts of *Psalliota xanthoderma* and *Cortinarius rotundisporus*.

The substance responsible for activity was probably different for each fungus as differences were evident between the two extracts in relative strength of activity against various bacteria and in lability to heat and other agents.

The activity of both extracts was unaffected by the presence of 10 p.c. of serum.

The toxicity of crude preparations was low.

Acknowledgments. I wish to thank Professor J. B. Cleland for identifying the fungi, and K. Mary Draper and Helen Chibnall for performing some of the tests.

REFERENCES.

Cleland, J. B. (1934): "Toadstools and Mushrooms and other Larger Fungi of South Australia", Part 1, Government Printer, Adelaide.

Heatley, N. G. (1944): Biochem. J., 38, p. 61.

THE STRUCTURE AND ELASTICITY OF THE CORTEX OF KERATIN FIBRES

AN ELECTRON MICROSCOPE STUDY

by E. H. MERCER AND A. L. G. REES

(From the Division of Physics, C.S.I.R., National Standards Laboratory, Sydney, and the Division of Industrial Chemistry, C.S.I.R., Melbourne).

(Accepted for publication 12th June, 1946.)

Certain conclusions concerning structural formulae drawn from the study of keratin fibres (Astbury, 1940) have had far-reaching consequences in discussions on the structure of proteins. The formulae concerned have been deduced from considerations involving the X-ray diffraction and the extensibility of *whole* fibres It is desirable that these deductions should be confirmed by the study of component parts of these heterogeneous fibres. With this end in view Woods (1938) has demonstrated that the cells of the fibre cortex, which is recognized as the elastic element of the fibre, duplicate very closely the properties of the intact fibre. The cortical cells are not however homogeneous. The optical microscope shows that they consist of an apparently homogeneous matrix in which are embedded longitudinally aligned fibrils. In this paper we shall describe some electron-microscopical observations, on the sub-cellular structure of the cortex, paying particular attention to evidence relating to the elastic properties of the sub-cellular components.

The material used in this study was a fine non-medullated (70's) Australian merino wool.

It is necessary to reduce such material intended for electron microscopy to a small size, and we have used both mechanical and enzymic means to disintegrate the fibre into cells and cellular fragments. The details of these procedures have been described elsewhere (Mercer and Rees, 1946).

The electron miscoscope used was an R.C.A. Type E.M.U.

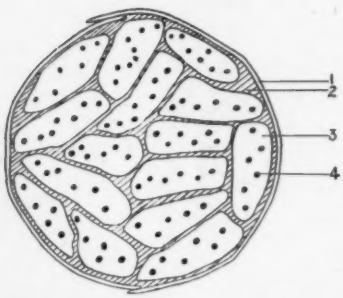


Fig. 1. Idealized cross-section of fine merino wool showing corticular structure: (1) cuticle, (2) intercellular cementing substance (somewhat exaggerated), (3) inter-fibrillar matrix, (4) intra-cellular fibrils.

DESCRIPTION OF ELECTRON MICROGRAPHS.

Optical microscopy has shown that the cortex of the wool fibre consists of long thin spindle-shaped cells (100μ long) held together by an intercellular cement (presumed a variety of keratin). An idealized cross-section of a fine wool fibre derived largely from the evidence of optical microscopy is given in Fig. 1. The scaly cuticle is of little significance in determining the elastic properties. The cross-sectional arrangement of the cortical cells in fine merino wool shows no symmetry; in hair, however, a concentric arrangement is found.

The cells may be isolated by a mechanical grinding process, which tears them apart, or by digestion with an enzyme (trypsin or pepsin), which removes the intercellular cement. When examined in the *electron microscope* (E.M.) the cells produced by the two processes differ in appearance. The tryptic-isolated cells are smooth surfaced, denser fibrils, and a less dense interfibrillar material being frequently observed within the cells (Fig. 2); whereas

the mechanically disintegrated cells have an uneven surface as may be seen in Fig. 3. After digesting the mechanically disintegrated cells for a few hours in trypsin their appearance resembles that of the cell in Fig. 2.

When the isolated cells are subjected to further mechanical grinding they break up into fibrillar fragments by longitudinal fission. In such preparations two types of material predominate: (1) dense fibrils of width 1,000–2,000 Å (0·1 to 0·2μ) (Figs. 4 and 5); and (2) a less

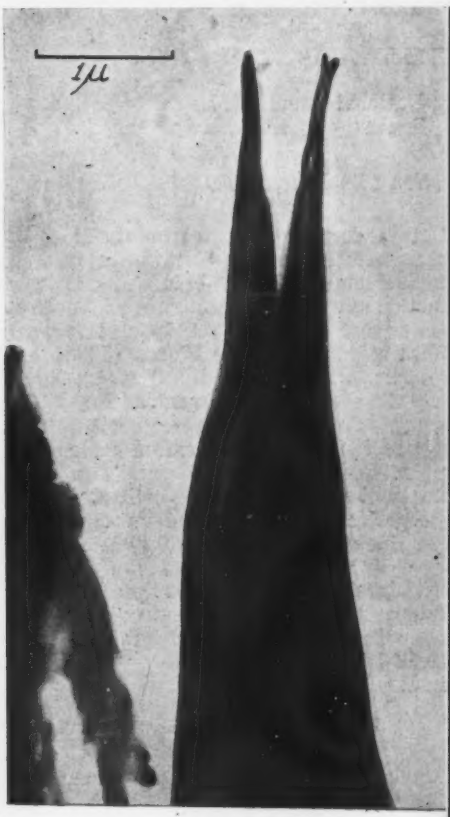


Fig. 2. End portion of cortical cells isolated from wool by the action of trypsin. The bifurcated end is common in merino wool.

Fig. 3. Portion of cortical cell isolated by mechanical action. The uneven surface of this preparation is to be contrasted with the smooth surface of the cell shown in Fig. 2.

Fig. 4. Corticular fragment showing presence of (a) intra-cellular fibrils, and (b) intra-fibrillar matrix.

dense inter-fibrillar component (most obvious in Fig. 5). Since the inter-fibrillar material is apparently digested by trypsin the appearance of the cell fragments also depends on the degree to which the matrix has been removed. Occasionally the surface of the specimen indicates the presence of particles of diameter ranging from a lower limit of about 140 Å (possibly molecular) to several hundred Å.

Zahn (1943) and Hock and McMurdie (1943) have both previously described the appearance of the fibrils in the E.M. and have found it convenient to distinguish also micro-fibrils, which are produced apparently by further longitudinal fission of the fibrils. In preparations produced by tryptic digestion the tips of some of the cortical cells were found to be formed by fibrils extending from the body of the cell, and tapering from about 2,000 down to a few hundred Å (Fig. 6). Since these fine tapering tips cannot be seen optically when less than 2,000 Å wide, the

true length of the cortical cell is actually longer than reported by optical examination. It is probably at least 120μ long. By a fortunate rupture of the supporting Parlodion film it was sometimes found possible to observe the stretching of fine fibrils under the E.M. They were found to be more extensible than whole fibre; in fact, the fibrils shown in Fig. 7 have stretched to about three times their original length as can be seen from the relative diameters of the elongated portions and the varices of initial width. When rupture occurs contractions amounting to about 50 p.c. of the length prior to rupture have been observed. This behaviour of fragments,

Fig. 5. Corticular fragments similar to Fig. 4 further broken up by grinding. In both Fig. 4 and Fig. 5 the greater part of the matrix has been removed by trypsin. In the background the thin supporting film may be seen to be ruptured in places.

which under the evacuated conditions of the microscope must have a very low water content, contrasts remarkably with the fact that dry whole fibre can be stretched only about 20 p.c. before rupture.

As one would expect, the presence of an inter-fibrillar component is more obvious in mechanically-disintegrated samples. The most striking electron micrographs illustrating its properties were obtained by reducing the wool with sodium thioglycollate at pH 7, dialysing until free from salts and then mechanically disintegrating the wool in a small ball mill in the presence of water. The thioglycollate treatment increases the extensibility of the wool by reducing the

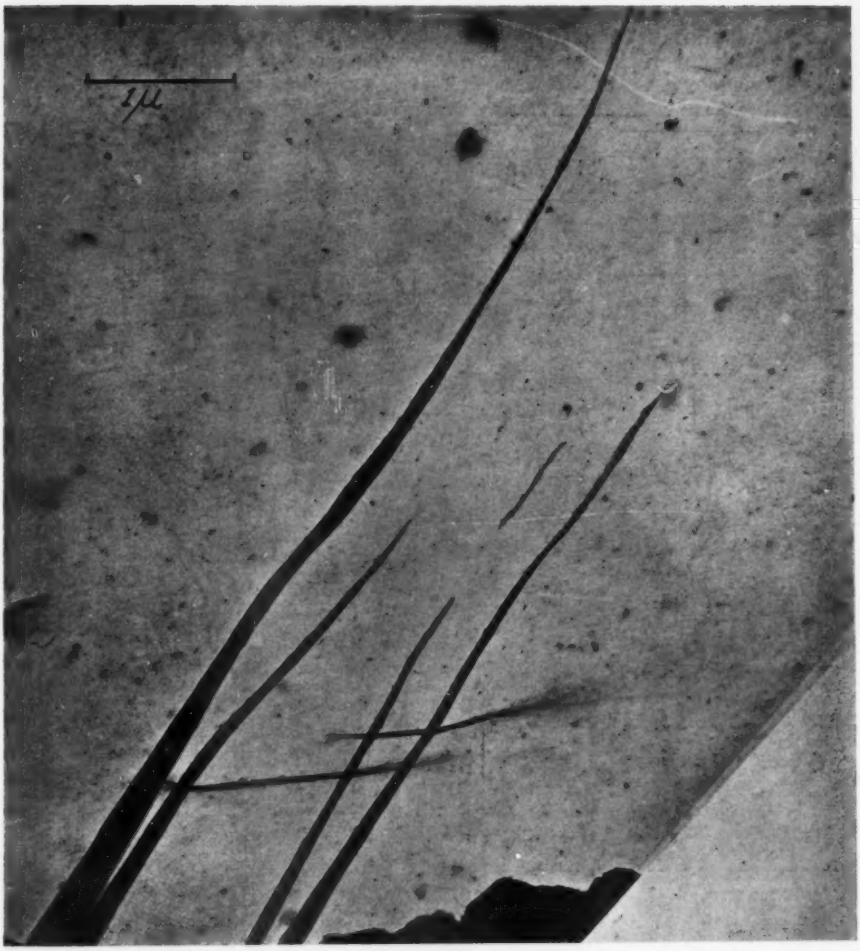


Fig. 6. Fibrils extending from ends of cortical cells. The adhering matrix has been removed by enzymic action. The background is spotted with colloidal particles presumably derived from the dispersion of the matrix by grinding the sample in water.

disulphide linkages (Patterson, Geiger, Mizell and Harris, 1941). The inter-fibrillar material in this type of preparation has the appearance of a viscous gum-like material adhering to the denser fibrils (Fig. 8). Its extensibility even under the anhydrous conditions of the microscope is very marked. Long, thin threads may be drawn from it, which on rupture sometimes contract as much as 50 p.c. of their length before rupture and then remain rigidly extended (Fig. 9).

INTERPRETATION OF ELECTRON MICROGRAPHS (E.M.Gs.).

The E.M.Gs. confirm the existence of a sub-cellular structure of longitudinally aligned fibrils embedded in a matrix of apparently amorphous material as already

deduced from optical methods (see Fig. 1). The fibrils are longitudinally striated and can be split into micro fibrils as fine as 200 Å wide. It seems certain that most of the anisotropy of the fibre and the oriented X-ray patterns must be due to the fibrillar component. The sub-cellular matrix and the intercellular cement, however, together constitute the greater proportion, perhaps amounting to 70 p.c. of

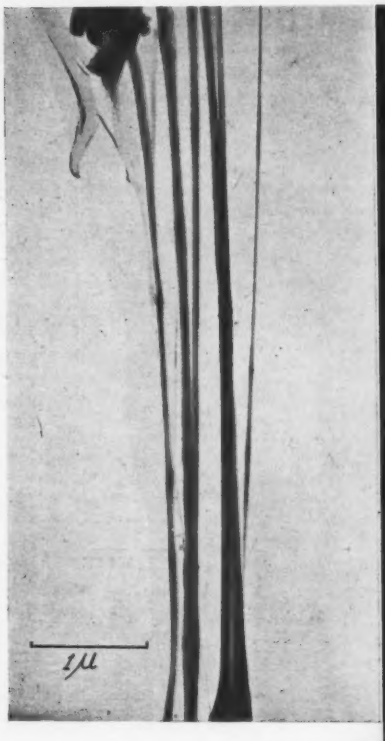


Fig. 7. Fibrils stretched by rupture of the supporting film. Portions of the film may be seen adhering to one end of the group of fibrils.

Fig. 8. Cellular fragments from "reduced wool" showing large fragments and interfibrillar matrix.

the total volume of the fibre, (Fig. 1 (2) and (3)). Their properties are in strong contrast to those of the fibrils. The inter-fibrillar component appears amorphous and solid, but under stress it may be distorted and drawn out into threads in a manner that strikingly resembles the behaviour of a viscous rubber solution (Figs. 5, 8 and 9). When a thread breaks it exhibits some contractility but rapidly sets into a solid form, which is rigid and self-supporting even in threads 500 Å wide. These properties appear to indicate a gel-sol transformation of this phase. The solid material under stress appears to pass from a rigid solid into a viscous sol,

but gels again when the stress is removed. These changes were observed in high vacuum and in the absence of water. Water increases the extensibility of whole keratin fibres and it is reasonable to expect, therefore, that the elastic behaviour of the components would be even more marked in the presence of water.

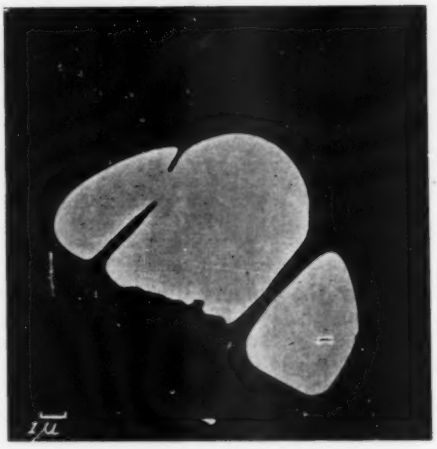


Fig. 9. Recovery after rupture of a thread of "reduced" matrix.

The extension of the cementing material between the cortical cells, which is seen as an uneven layer on the surface of mechanically disintegrated material (Fig. 3) was not actually observed. There are some indirect reasons for concluding that it is broadly similar in properties to the inter-fibrillar phase. In the optical microscope their refractive properties are similar; both are digested by trypsin whilst the fibrils are not, and the appearance of the surfaces in Fig. 3 could be accounted for by assuming the cement had been drawn out in threads and partly recovered on rupture to form the nodular structures seen on the surfaces in the micrographs of mechanically disintegrated wool.

On the basis of their X-ray evidence Astbury and Woods suggested the existence of a graded series of "well and less well organized" regions within the cortex. The E.M. enables us to take a further step and make a more definite division into fibrous and amorphous regions[1]. There is no evidence for *discrete* micelles, 200 Å. thick and about ten times as long, such as were postulated by Speakman (1931) to explain his experiments on swelling, but the longitudinally extended structure of fibrils could be utilized to explain the swelling and absorption data.

The fibrils seen in the optical microscope appear to form a network structure (Mercer, 1942; Reumuth, 1942). E.M.Gs. such as Fig. 5 also show fibrils crossing and overlapping, and when the inter-fibrillar matrix has been partially removed they may be found held together in places by remnants of matrix giving a superficially net-like appearance.

THE ELASTICITY OF WOOL.

A structure of fibrils embedded in a viscous matrix has already been postulated as a basis for an explanation of the elastic properties of wool fibres (Speakman, 1931), but was abandoned in favour of the current "folded molecular chain" theory (Astbury and Woods, 1933). More recently Bull and Gutmann (1944) have considered that a development of the earlier theory is required to explain their elasticity experiments.

The stress-strain curve of wool and hair in its normal condition is characterized by a yield point at about 2 p.c. extension followed by a phase of rapid extension with the development of

[1] It is possible to deduce the existence of a fibrous and an amorphous phase from the X-ray photographs of wool and derived proteins. The fibrous phase is characterized by the imperfect fibre diagram discussed by Astbury and Street; the amorphous phase by the diffuse ring system 4·2Å (2·1 and 1·05Å also occur), 10Å and 27Å. This question will be discussed in a further communication by us.

greater resistance to stretching between 20 and 30 p.c. extension. Speakman originally conceived the earlier stage of extension (0–20 p.c.) to be accompanied by the straightening of the fibrils and the increasing resistance after 20 p.c. to be due to the added resistance of the now taut fibrils. Two main objections have been raised against this theory:

(1) the fibrils in unstretched wool are already well aligned and only about 10 p.c. extension would be possible before they were drawn taut; and

(2) a re-alignment of fibrils sufficient to yield even 20 p.c. extension should be detectable in the X-ray patterns which, however, show no very marked change in the region of 0–20 p.c.

The discovery that stretching produced a change in the X-ray diffraction pattern led Astbury and Street (1931) to the view that the opening of a folded molecular chain accompanied fibre extension. The two fundamental assumptions implicit in their theory are:

(i) the X-ray diffraction patterns yielded by the relatively small proportion of the fibre (wool or hair), which is crystalline and oriented, may be used as a clue to the structure of the less precisely organized portions of the fibre; and

(ii) the maximum attainable extension of the whole fibre (100 p.c.) is a demonstration that the component molecules are folded in such a way that they also double their length on stretching.

Structural formulae (Astbury and Street, 1931; and Astbury, 1941) have been devised which exhibit the required molecular spacings and which double their length when unfolded.

E.M.G.'s obtained in this study show that the primary division of the subcellular cortex is into the fibrous and amorphous phases. Within the fibrous phase itself imperfection of structure may be indicated by the lack of exact parallelism and alignment of the micro-fibrils. When the whole fibre is stretched, portions of the matrix probably stretch in the same proportion, but in view of the amorphous character of the matrix it cannot be concluded that its component molecules extend in the same proportion. There is, therefore, no confirmation of either of the two basic assumptions mentioned above.

The $\alpha \rightarrow \beta$ transformation of the X-ray patterns becomes apparent beyond 20 p.c. fibre extension, and according to Astbury and Woods (1933) is complete at 70 p.c. fibre extension. The actual transformation therefore takes place as the crystalline phase is stretched about 40 p.c. in length. Without the two assumptions mentioned above we could hardly conclude that this involves the doubling of the chain lengths. The fibrils can, as we have seen, actually stretch beyond 100 p.c., but there is no evidence that in the whole fibre they extend so far before the fibre breaks.

These results can however, be brought into harmony with a modified form of Speakman's earlier theory. A picture of the sequence of events during stretching in which consideration is given to the rôle of the matrix is as follows:

(a) 0–2 p.c. The fibre extends as a rigid body, Hookes' Law is obeyed and, as shown by Astbury and Woods, intermolecular spacings throughout increase by 2 p.c.

(b) 2–20 p.c. At 2 p.c. the yield point of the intercellular and intra-fibrillar gel is reached and flow occurs in this phase. At 20 p.c. extension of the fibre the cortical cells have increased in length by 10 p.c. only (Woods, 1938). Ten p.c. of the stretch must therefore be taken up in the *inter*-cellular cement, which, we have argued, has similar sol-gel properties to the intra-cellular matrix. The extension of the cortical cells is effected by a flow within the viscous (sol) matrix accompanied by straightening of the fibrils. A slight sharpening of the α-pattern is said to occur in this region and this may well be ascribed to the straightening of the fibrils.

(c) Beyond 20 p.c. both the fibrils and the matrix are stretched together with an increasing resistance to extension developing owing to the greater yield value of the fibrils. The fibrils presumably contain the fibrous X-ray diffracting material and its extension produces the changes in the X-ray pattern. The 40 p.c. extension of the fibrils, which takes place as the α-pattern is replaced by the β-pattern, may be effected by the unfolding of molecular chains. Huggins (1943) has

suggested methods of folding polypeptide chains which allow of this order of extension.

The change from gel to sol of the matrix is assumed to be produced by the rupture of linkages such as the salt and disulphide. Their re-formation converts the sol to a gel again giving rise to the phenomenon of "set".

SUMMARY.

An electron microscopic study of fragments, derived from the cortex of wool fibres by enzymic and mechanical disintegration, reveals the presence of two main components (1) a fibrillar, and (2) an amorphous matrix.

Some observations on the extensibility of these components have been possible. The matrix in particular flows under stress like a viscous liquid.

The sequence of events during the stretching of wool is discussed.

Acknowledgment. We wish to thank Miss H. P. Meggs for preparing the drawing for Fig. 1.

REFERENCES.

Astbury, W. T. (1940): Trans. Far. Soc., 36, p. 871.
Astbury, W. T. (1941): Nature, 147, p. 696.
Astbury, W. T. and Street, A. (1931): Phil. Trans. Roy. Soc. (London), A 230, p. 75.
Astbury, W. T. and Woods, H. J. (1933): Phil. Trans. Roy. Soc. (London), A 232, p. 333.
Bull, H. B. and Gutmann, M. (1944): J. Amer. chem. Soc., 66, p. 1253.
Hock, C. W. and McMurdie, H. F. (1943): J. Res. Nat. Bur. Stand., 31, p. 229.
Huggins, M. L. (1943): Chem. Rev., 32, p. 195.
Mercer, E. H. (1942): J. Counc. sci. ind. Research (Aust.), 15, p. 221.
Mercer, E. H. and Rees, A. L. G. (1946): Austral. J. exp. Biol., 24, p. 147.
Patterson, W. I., Geiger, W. B., Mizell, L. R. and Harris, M. (1941): J. Res. Nat. Bur. Stand., 27, p. 89.
Reumuth, H. (1942): Melliand Textilberichte, 28, p. 1.
Speakman, J. B. (1931): Proc. Roy. Soc. (A), 132, p. 167.
Woods, H. J. (1938): *Ibid.*, 166, p. 76.
Zahn, H. (1943): Textilberichte, 24, p. 157.

CLOTTING IN HEART AND BRAIN

(EXPERIMENTAL STUDY)

by E. R. TRETHEWIE

(From the Institute of Medical and Veterinary Science, Adelaide, and the Department of Experimental Medicine, University of Adelaide).

(Accepted for publication 29th April, 1946.)

It has been shown that when diluted heparinized blood (0·05 mg. heparine (Kahlbaum) per c.c.) is perfused through the kidney and liver the perfused fluid will not coagulate, or coagulation is only very slight even after 24 hours, while the control, unperfused heparinized blood readily clots. The lung was less active, but still markedly inhibitory. It was also shown that after the diluted plasma obtained from the liver had been extracted with ether and chloroform-ethanol the protein residue inhibited coagulation of other coagulable heparinized blood (Trethewie, 1945, a, b). It was suggested that the anticoagulant substance concerned might be the one extracted from tissues by De Sütö-Nagy (1944) which is a lipo-protein.

This paper concerns the perfusion of the livers and hearts of dogs and cats and the head of the cat with diluted blood in an endeavour to see if there is any fundamental reason why clotting is liable to occur in the heart and brain additional or complemental to vascular changes, since marked vascular changes may be seen elsewhere and yet there is a preponderance of clot development in heart and brain, compared with other organs.

METHOD.

Diluted Blood Perfusions.

The perfusions were performed on dogs and cats under nembutal anaesthesia (70–115 mg. per kilo injected intraperitoneally). For dogs 1,500 c.c. diluted blood were used, and for cats 500 c.c. The organs appeared normal macroscopically before perfusion.

Liver. As described previously (Trethewie, 1945b), the liver was perfused via the portal vein with the animal's own blood which had been diluted in 9 times its volume of Tyrode and contained 0·022–0·066 mg. heparine (Kahlbaum) per c.c. Except in one instance (D8) the whole liver was perfused. The gall-bladder was removed.

Heart. The heart was perfused through the aorta after excision. The oxygenated, heparinized blood used for perfusion was diluted in 9 times its volume of a Locke solution (sodium chloride 9·0 gm., potassium chloride 0·42 gm., calcium chloride 0·24 gm., sodium bicarbonate 0·1 gm., dextrose 1·0 gm., distilled water 1,000 c.c.). Perfusion was begun with the Locke solution and then continued with diluted blood. The perfused blood was collected in a beaker placed directly beneath the heart. The rate of flow was regulated by a screw-clip.

Head. Heparinized blood diluted with Tyrode (1 volume blood: 9 volumes Tyrode) was used. A cannula was introduced into the ascending part of the aorta which delivered the diluted blood to the head. Perfusion was begun with Tyrode and then continued with diluted blood. The aorta was tied off caudal to the left subclavian artery, the axillary vessels were tied off as were the internal mammaries. The superficial vessels in the neck were divided and ligated caudally. A cannula was placed in the superior vena cava to collect the perfused sample. The head of pressure used in this and in the preceding type of perfusion was 140 cm. of diluted blood. The rate of flow was regulated by a screw-clip.

After the perfusion had been continued for a quarter of an hour the diluted, perfused blood was collected in a beaker (S_3). At the beginning and at the end of the perfusion diluted blood was taken by a dry syringe from the rubber tubing proximal to the cannula (S_1 and S_2 respectively). The apparatus was agitated regularly to avoid sedimentation.

Assessment of Clotting.

1·8 c.c. of blood from each sample was placed in a bottle (2 cm. diameter) and observed for clotting. Readings were made at 24 hours (temperature 11–13° C.). A large clot was classified as ++, small clots +, a trace ±, and clot-free —.

Heparine Assay.

Heparine was assayed by a modification of the method of Copley and Whitney (1944), as described previously (Trethewie and Melvin, 1945). Heparine was estimated as heparine (Kahlbaum), though in cases heparine (Boots) was used and it was found by the method employed using our material that heparine (Boots) 1 mg. = 4 mg. heparine (Kahlbaum) approx.

Prothrombin Assay.

Prothrombin was assayed by a modification of Quick's (1942) method as described previously (Trethewie, 1945b). This modification was necessary since the blood was heparinized. The percentage of prothrombin was recorded as compared with a standard human plasma taken as 100 (Fig. 1), and the graph was prepared from estimations of the prothrombin time of serial dilutions of this plasma in saline.

EXPERIMENTAL.

It has been shown that the normal liver may be perfused with diluted heparinized blood (0·05 mg. heparine per c.c.) and, after perfusion, the perfused fluid does not coagulate, or coagulates only slightly, after 24 hours, while the control, unperfused, heparinized blood coagulates markedly. This was the finding in every one of eight liver perfusions. Further, the protein sample of the perfused fluid will inhibit coagulation of other coagulable heparinized blood, while that of the unperfused will not (Trethewie, 1945b). Now we may vary the conditions by reducing the heparine content of the blood, and so may obtain coagulation of the perfused fluid, e.g. in the case of D7 (Table 1) the heparine content was reduced to half and then the perfused fluid did show clots though not as large as in the case of the control blood. A variation may also be produced by varying the rate of perfusion. If the rate of flow is fairly rapid one might expect little anticoagulant property to be given to the blood per unit vol. This is not as important as one might expect, probably because with a slow rate of perfusion less of the organ actually perfuses.

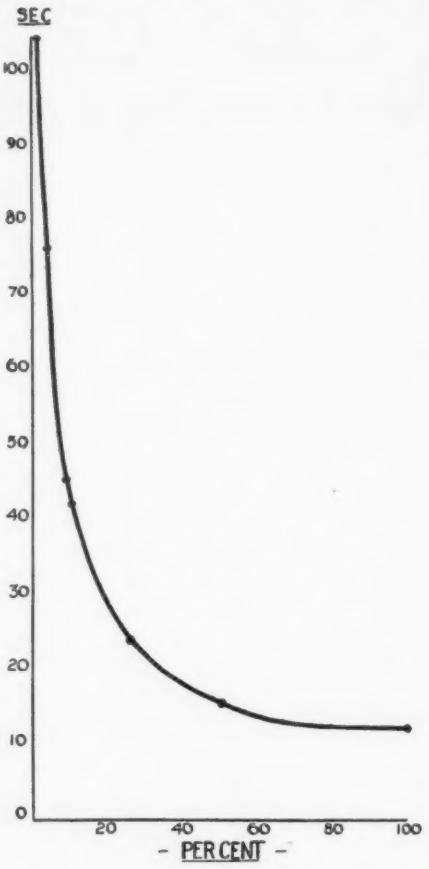


Fig. 1. Graph relating percentage prothrombin (selecting a normal blood = 100) with prothrombin time. Technique as described in method. Ordinate: prothrombin time in sec. Abscissa: percentage of plasma diluted in saline.

We may then determine the rate of perfusion of the liver (per gm. organ per unit time) and the heparine content of the perfusing fluid in perfusions where the blood develops anticoagulant properties. In this range we may perfuse the heart with sufficient blood to keep the heart beat active. When we perfuse the heart at a rate comparable to the liver the blood perfused through the heart does clot, and equally or almost as much as does the control blood.

In the case of the head perfusion the rate of flow is given as though all of the blood had passed through the brain. This is not quite the case, but in view of the method employed it would only be a little higher than the true figure, thus we are certain that the findings are not due to too rapid a rate of perfusion, because even the figure given is below that of the perfusion rate

for the liver which will confer anticoagulant effects upon the perfused fluid. In the case of the head the perfused fluid which contains the standard amount of heparine will clot almost as much as the control fluid and in one experiment clotted while the control fluid did not clot. These findings are detailed below.

TABLE 1.

No.	Perfusion rate. c.c./gm. organ/hour.	Added heparine. mg./c.c.	Clotting.* S_1	S_2	S_3
		Heart.			
D1	12	0·05	+	+	+
D2	8	0·05	+		<+
	15			++	++
C3	29	0·05	+	+	+
C4	31	0·05	++	++	<++
		Head.			
C5	10	0·044	+		<+
	3			+	<+
C6	2	0·044	−		+
	12			−	−
		Liver.			
D7	16	0·022	++	++	+
D8	6	0·046	<++	<++	−
C9	36	0·05	++	++	±
C10	1	0·066	+	+	±

* ++ = large clot; + = small clots; < = a little less; ± = trace; − = no clot.

Liver Perfusion.

Cat. The rate of flow of the perfusion fluid in the earlier experiments (Trethewie, 1945b) was quite slow, approximately 1 cc./gm. organ/hour. In eight such cases, no clotting appeared in the perfused fluid (24 hours) in four and only a trace of clotting appeared in four. A trace indicates a very tiny clot (see Fig. 2: ±). The rate of flow was then much increased in a case where 0·05 mg. heparine per c.c. was added to the diluted blood (C9, Table 1). Here the rate of flow was 36 cc./gm./hour and the control fluid showed a large clot while only a trace of clotting appeared in the perfused fluid which was collected for 9 minutes. The perfused fluid was xanthochromic.

Dog. When a dog liver was perfused at a rate of 6 c.c./gm./hr. (Table 1, D8) for 30 minutes the control fluid showed large clots while the perfused fluid did not clot (Fig. 3). This experiment was performed on a piece of liver weighing 63·5 gm. and 0·046 mg. heparine per c.c. was used in the perfusion fluid. In another experiment the whole liver was perfused for 19½ minutes and only 0·022 mg. heparine per c.c. was added. This was insufficient to allow much inhibition of clotting and the rate of perfusion was 16 c.c./gm./hour (Table 1).

In view of the similarity of the findings with a perfusion rate of 1 to 36 c.c./gm./hour, provided the heparine content is 0·046–0·066 mg. per c.c., a liver was injected via the cannula with 1 c.c. of coloured fluid while perfusing at a rate of 6·5 c.c./gm./hour and then injected similarly on increasing the rate to 53·3 c.c./gm./hour. There was a much greater area of liver injected in the second injection. This is shown in Fig. 4. A lobe is outlined with an interrupted line and at (a) the injected area is shown by the heavy line when perfused at 6·5 c.c./gm./hour and at (b) the injected area is similarly shown when perfused at 53·3 c.c./gm./hour.

Heart Perfusion.

Dog. The hearts of three dogs were perfused. In one heart from a dog weighing 5·3 kg., 0·1 mg. heparine per c.c. was added to the perfusion fluid and the heart perfused for 10 minutes at 33 c.c./gm./hour. Neither the perfused nor the control fluid clotted. This was probably due to the high heparine content. In the two other instances 0·05 mg. heparine per c.c. was added to the fluid before perfusion and the control fluid in the one produced small clots and the perfused sample clotted similarly. Here the perfusion rate was 12 c.c./gm./hour. In the other the heart was perfused at 8 c.c./gm./hour for 16 minutes. The first control sample produced

small clots and the perfused fluid clots almost as large. The perfusion rate was increased to 15 c.c./gm./hour for 41 minutes and the control sample of fluid produced a large clot. The perfused sample clotted similarly.

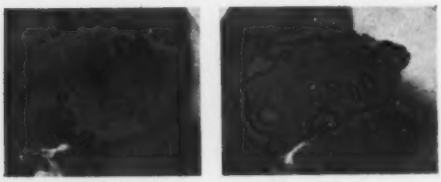
Cat. Two cat hearts were perfused with fluid containing 0·05 mg. heparine per c.c. In the one the heart was perfused at 29 cc./gm./hour and equal clotting appeared in both control and the perfused sample. In the other the heart was perfused at 31 c.c./gm./hour for 32 minutes and a large clot developed in each control sample and a slightly smaller clot in the perfused sample.

1 2 3

Fig. 2. Appearance at 24 hours of clot from equal amounts of control blood (1, 2) and blood perfused through a liver (3). 1 and 2 are denoted as ++, 3 as ±. Details in text.

1 2

Fig. 3. Appearance of blood. 1, control blood. 2, blood perfused through liver. Readings at 24 hours. Further details in text.



(a) (b)

Fig. 4. Appearance of a lobe of liver following injection of 1 c.c. of coloured fluid when perfused at a rate of 5·6 c.c./gm./hr. (a), and at 53 c.c./gm./hr. (b). Lobe outlined with interrupted line, perfused areas outlined with continuous line. Details in text.

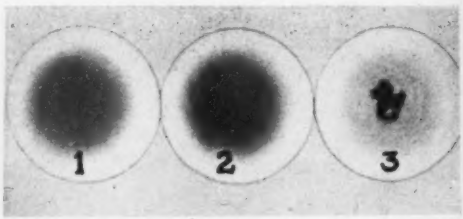


Fig. 5. Appearance of control blood (1, 2) and perfused blood (3) perfused through the head. Readings made at 24 hours. Details in text.

Head Perfusion.

The heads of three cats were perfused. In one from a cat weighing 3·3 kg., 0·3 mg. heparine per c.c. was added to the diluted blood and the brain perfused at 7 c.c./gm./hour. Neither control sample nor perfused sample clotted, probably because of the excessive amount of heparine used. In another cat the brain was perfused at 10 c.c./gm./hour, 0·044 mg. heparine per c.c. added and the control fluid produced small clots. The perfused fluid collected for 35 minutes produced clots of nearly the same size. The perfusion rate was slowed to 3 c.c./gm./hour and the control sample of fluid and the perfused sample collected for four minutes produced similar clots. The head of another cat was perfused with fluid containing 0·044 mg. heparine per c.c. at 2 c.c./gm./hour and the control sample of fluid did not clot. The perfused sample collected for 33 minutes produced small clots (Fig. 5). The perfusion rate was increased to 12 c.c./gm./hour and the control sample and the perfused sample collected for 13 minutes did not clot.

Prothrombin Content of Perfused Fluid.

When endeavouring to determine the cause of non-coagulation of blood perfused through the liver estimations were made of the prothrombin content as was recorded in a previous paper, and though in the organs perfused here (except the liver) the anti-coagulant property was not evident, in some cases estimations were made of the prothrombin content of the perfusion fluid. In the case of the liver (Trethewie, 1945b) it was found once in five cases that there was a fall

in the prothrombin content of the perfused fluid. In the remaining cases there was little alteration. In D7 (Table 2) a similar estimation showed a concentration of 14½ p.c. prothrombin in the perfused fluid compared with 14 p.c. in both the controls. In D8 there was much less prothrombin, again with little change in the content of the perfused fluid. Estimations of prothrombin in the dog will not necessarily give an estimate of the amount of prothrombin compared to normal for the dog since we are comparing it against a human plasma (standard 100).

In the case of the cat heart little alteration was found in the prothrombin content (Table 2).

TABLE 2.

No.	Heparine. mg./c.c.			Prothrombin time. (sec.).			Prothrombin. p.c.		
	S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
				Heart.					
C4	0·045	0·038	0·032	47·7	43·9	41·2	8	9	10
				Head.					
C5	0·044		0·045	85·7		86·6	4		4
		0·045	0·044		84·3	95·3		4	4
C6	0·044		0·04	183·3		71·0			4½
		0·044	0·04		189·3	90·1			4
				Liver.					
D7	0·022	0·022	0·018	36·8	37·2	35·5	14	14	14½
D8	0·045	0·044	0·04	269·0	283·0	312·7			

In the case of the head perfusion, in one experiment there was little alteration in the prothrombin content while in the other (C6) it was found that the prothrombin content of the perfused fluid was higher than that of the control. This probably accounts for the non-coagulation of the control fluid while the perfused fluid clotted. It seems not unlikely that something was added to the fluid after passing through the brain.

Heparine Content of Perfused Fluid.

Estimations of the heparine content of the blood were comparable with what had been found previously in the case of the liver (Trethewie, 1945b). In general there was a fall in the amount in the fluid after perfusion compared with the control, e.g. in the case of the heart (C4) the perfusion fluid contained 0·045 and 0·038 mg. heparine per c.c. in the control fluids and 0·032 mg. heparine per c.c. in the perfused fluid. In the head perfusion the fall was less evident. In one experiment there was no change (C5) and in the other the control fluid contained 0·044 mg. heparine per c.c. and the perfused fluid contained 0·040 mg. heparine per c.c. (Table 2). In the case of the liver perfusions there was little change in the heparine content of the perfused sample in one experiment and a slight fall in the other (Table 2).

DISCUSSION.

It has been shown in previous papers (Trethewie, 1945, a, b) that the perfused lung, kidney and liver release some substance into the vessels which inhibits coagulation. The lung is not so active in this regard as the liver or kidney. It was suggested that this substance might be important in maintaining the fluidity of the blood. The heart and the head when treated similarly do not endow the blood with anticoagulant effects: in fact in one instance blood after leaving the head showed an increased coagulability. It is therefore suggested that there is a mechanism in some organs of the body whereby inimical effects causing coagulation are reduced. A slowing rate of blood flow has been considered an important factor in the development of thrombosis, but it would appear that the liver can overcome this activity by passing anticoagulant into the blood. It is certainly rare to find thrombosis in the liver, and the work recorded here shows some basis for this finding. The kidney is rarely the seat of recognizable thrombosis. We find fibrosis in a kidney which is the seat of arterial disease, but thrombosis recognized as such is very rare. It seems the explanation may be that the kidney supplies much anticoagulant to the blood as has been shown to be the case (Trethewie, 1945 b).

The heart is frequently the seat of arterial disease. It is also a favoured region for the development of thrombosis. It is suggested that the inability of the heart to liberate anticoagulant may be important for this. It is not infrequently the case that a patient presents himself with symptoms suggesting a sudden onset of coronary thrombosis, and yet at autopsy no gross recent thrombus is found. Frequently an obstructive lesion of the coronaries is found which has been present for a considerable time. On the other hand no recognizable thrombus may appear (Wright-Smith, 1936). In a large series Wright-Smith found a thrombus in a coronary artery or its branches in only one case in eleven. It is possible that such cases have thrombi in the smaller vessels which are not detected except after careful investigation and Wright-Smith discusses this. Atheroma of the coronary vessels will impair the blood supply to the heart, and the degree of impairment is related to the level of the blood pressure. In gelatine infusions it has been shown that the area supplied increased with increased pressure (Lowe, 1941). Anastomotic channels may be very important in supplying blood if the pressure differences across them is great. Short of obstruction there will be an impaired flow through the capillaries if the arterial lumen is narrowed. Now if the heart is at a disadvantage with regard to passing anticoagulable substance into the blood it is not inconceivable that an impaired flow through this organ may allow clotting to occur under conditions in which it would not occur elsewhere, e.g. in the liver where much anticoagulant substance is released. The brain likewise is commonly the seat of thrombosis and a similar explanation may be responsible. In addition it is noted that in one experiment with a slower rate of flow the blood flowing from the head was more coagulable than control blood.

It is also frequently found that symptoms suggestive of vascular disease in the brain occur where thrombotic changes are not found in the larger vessels, and many consider that the lesion has affected the smaller vessels; and if so it is possible that the mechanism is related to the absence of output of anticoagulant substance. It is not unlikely the substance is poured out in the small vessels, since organs differ in their ability to inhibit coagulation while the larger vessels in these experiments were of similar healthiness.

SUMMARY.

Perfusion of the liver of the dog and cat with diluted heparinized blood (0·044 mg. per c.c.) endows the perfused fluid with increased anticoagulant activity.

Perfusion of the heart of the dog and cat with diluted blood similarly heparinized does not produce this effect. Perfusion of the head of the cat with diluted heparinized blood is similarly ineffective.

The significance of these findings is discussed.

REFERENCES.

Copley, A. L. and Whitney, D. V. (1944): J. lab. and clin. Med., 29, p. 117.
De Sütö-Nagy, G. J. (1944): J. biol. Chem., 156, p. 433.
Lowe, T. E. (1941): Amer. Heart J., 21, p. 326.
Quick, A. J. (1942): The Hemorrhagic Diseases and the Physiology of Hemostasis, Thomas, Spingfield, Ill.
Trethewie, E. R. (1945a): Austral. J. exp. Biol., 23, p. 231.
Trethewie, E. R. (1945b): Med. J. Austral., 2, p. 328.
Trethewie, E. R. and Melvin, P. A. (1945): Austral. J. exp. Biol., 23, p. 241.
Wright-Smith, R. J. (1936): Royal Melbourne Hosp. Clin. Reports, 7, p. 71.

INACTIVATION OF VACCINIA AND ECTROMELIA VIRUS HAEMAGGLUTININS BY LECITHINASE

by JOYCE D. STONE[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 18th June, 1946.)

A striking resemblance exists between the haemagglutinating properties of the vaccinia and ectromelia virus haemagglutinins and of certain mixtures of tissue lipids, more particularly those containing a phospholipid (Burnet and Stone, 1946; Stone, 1946). Both classes of haemagglutinins react with essentially the same, highly characteristic range of red cells which includes those from a proportion only of fowls. The fact that the haemagglutinins produced by the vaccinia and ectromelia viruses are distinct from the virus particles themselves has recently been established (Burnet and Stone, 1946). It has already been suggested that these haemagglutinins are probably complexes of a phospholipid with a specific virus protein, the phospholipid conferring the capacity to agglutinate the unique range of red cells, the protein being responsible for its serological specificity.

On these grounds it was of interest to attempt inactivation of the haemagglutinins of the vaccinia and ectromelia viruses by the action of phospholipid-splitting enzymes. It has now been found that these virus haemagglutinins are readily inactivated by a preparation of *Cl. welchii* α toxin, presumably due to the action of lecithinase C (Macfarlane and Knight, 1941) and also by cobra venom (lecithinase A).

MATERIALS.

The *Cl. welchii* α toxin used in these experiments was kindly supplied by Dr. A. W. Turner of the Division of Animal Health and Nutrition, C.S.I.R. It was a dried preparation obtained by precipitation of type A cultures with saturated ammonium sulphate.

A stock solution of 2 mg. per ml. in normal saline was prepared and kept in the frozen state until required.

The cobra venom was a sample of dried material from Dr. C. H. Kellaway's collection of snake venoms. This was dissolved in normal saline 10 mg. per ml. and kept at 4° C.

Borate calcium buffer was prepared as described by McClean *et al.* (1943), and contained 0·3184 gm. borax, 2·061 gm. boric acid, 0·487 gm. sodium chloride per litre with 0·1 M calcium chloride. The pH of the undiluted buffer was 7·2. It was used in the tests in a final dilution of 1: 10 in normal saline.

Vaccinia virus haemagglutinin (V.H.A.) and ectromelia virus haemagglutinin (E.H.A.) were used in the form of suspensions of infected chorioallantoic membranes prepared allowing 0·5 ml. of saline per membrane (Burnet and Stone, 1946).

EXPERIMENTAL.

Susceptibility of V.H.A. to Inactivation by Cl. welchii *Toxin.*

Vaccinia virus haemagglutinin (V.H.A.) was incubated at 37° C. in a dilution of 1: 10 with toxin 1: 5,000 (0·2 mg. per ml.). Except where otherwise stated tests were performed in the presence of borate calcium buffer diluted 1: 10 so that the final concentration of calcium was 0·01 M. Samples taken at zero time and after incubation for varying periods were titrated in the usual manner with susceptible fowl cells (Burnet and Stone, 1946).

Treatment of V.H.A. with toxin under these conditions resulted in a progressive reduction of titre. The same concentration of toxin previously heated to 100° C. for 5 minutes as a solution in normal saline (2 mg. toxin per ml.) brought about no decrease in titre over a period of four

[1] This work was carried out under a grant from the National Health and Medical Research Council.

hours. There was evidence of slight inactivation by heated toxin when treatment was prolonged to 24 hours. Similarly, the inclusion of horse antiserum to *Cl. welchii* type A toxin in a dilution of 1: 10 completely inhibited the inactivation of V.H.A. by the toxin. Normal horse serum in the same concentration had no inhibitory effect. The results of tests demonstrating these findings are given in Table 1, tests numbers 1–5. In all Tables the haemagglutinin titres are expressed in terms of the test mixtures and should be multiplied by ten in order to read in terms of the original chorioallantoic suspension.

When the toxin was tested in the absence of calcium ions, there was no apparent destruction of V.H.A. For this purpose the test was performed in normal saline containing 0·2 p.c. sodium citrate. The borate calcium buffer was omitted. A comparison of the results of tests numbers 6 and 7 in Table 1 indicates the inactivity of toxin under these conditions. In the presence of borate calcium buffer and 0·2 p.c. citrate the toxin showed considerable activity (number 8, Table 1) only slightly less than in the test without citrate (number 2, Table 1). This concentration of citrate (0·006 M) was apparently inadequate to reduce the concentration of calcium ions present below the threshold level required for toxin activity.

TABLE 1.

Inactivation of vaccinia haemagglutinin (V.H.A.) by Cl. welchii *toxin.*

Test Number.	Components.	Haemagglutinin titre after incubation for the following times (in hours).			
		0	2	4	24
1	V.H.A.	40	35	30	25
2	V.H.A. + toxin	50	7	3	<1
3	V.H.A. + heated toxin (100° C. 5 minutes)	40	30	30	16
4	V.H.A. + toxin + antitoxin	40	35	35	35
5	V.H.A. + toxin + normal serum	50	14	7	< 1
6	V.H.A. + citrate	40	35	30	35
7	V.H.A. + toxin + citrate (borate calcium buffer omitted)	45	35	30	35
8	V.H.A. + toxin + citrate	40	10	7	7
9	V.H.A. + hyaluronidase (1 p.c. rat testis suspension)	40	30	28	25

All tests were carried out concurrently, using the same stock of V.H.A. Except for No. 7 all were made in the presence of 1:10 borate calcium buffer, i.e. 0·01 M calcium chloride.

It is well established (Macfarlane and Knight, 1941; Oakley and Warrack, 1941) that calcium or magnesium ions are required for the action of the α toxin or lecithinase of *Cl. welchii*, so that the complete inactivity of the toxin preparation against V.H.A. on the removal of these ions is strong evidence that it is the lecithinase which is responsible for its effect. However, as hyaluronidase is produced by a number of type A strains (Evans, 1943), the possibility of the action of the toxin preparation being in some way due to this enzyme was excluded by the following test. A 10 p.c. suspension of rat testis was prepared in saline as a source of hyaluronidase. When V.H.A. was treated with a 1: 10 dilution of this testicular extract no inactivation took place. The slight decrease in titre over a period of 24 hours was no greater than occurred with the control dilution of V.H.A. alone, as will be seen from a comparison of numbers 1 and 9, Table 1.

TABLE 2.

Blockade of Cl. welchii *toxin activity with lecithin.*

Components of test mixture.	Haemagglutinin titre after incubation for the following times (in hours).			
	0	1	2	4
V.H.A.	18	15	15	12
V.H.A. + lecithin	18	15	15	12
V.H.A. + toxin	20	5	4	3
V.H.A. + lecithin + toxin	18	10	8	5
Lecithin + toxin	<1	—	—	<1

Several attempts have been made to block the effect of the toxin on V.H.A. by the addition of lecithin. No striking results were obtained although in all experiments the presence of lecithin definitely retarded the destruction of V.H.A. Table 2 gives the results of a typical experi-

ment in which 1: 10 V.H.A. was treated with 1: 5,000 toxin in borate calcium buffer with and without the addition of lecithin in a concentration of 1: 500. The lecithin used was a purified preparation from ox heart kindly supplied by Dr. M. C. Pangborn. In order to avoid haemagglutination by lecithin itself (Stone, 1946) all titrations were carried out in normal rabbit serum 1: 10 which served to inhibit any nonspecific haemagglutination without significantly reducing the titre of V.H.A.

Action of Cl. welchii *Toxin on other Virus Haemagglutinins*

Ectromelia virus haemagglutinin (E.H.A.) was found to be similarly susceptible to inactivation by *Cl. welchii* type A toxin. It was tested as a 1: 10 dilution of a chorioallantoic membrane emulsion in the same way as described above for V.H.A. using a concentration of toxin of 1: 5,000 and borate calcium buffer as the test medium. It will be seen from the results in Table 3 that the control dilution of E.H.A. in the absence of toxin showed a 50 p.c. fall in titre over the incubation period of 4 hours. There was no increased inactivation in the presence of toxin previously heated to 100° C. for 30 minutes, while the fresh toxin caused a 95 p.c. reduction in titre over this period.

TABLE 3.

Inactivation of ectromelia haemagglutinin (E.H.A.) by Cl. welchii *toxin.*

Components of test mixture.	Haemagglutinin titre* after incubation for the following times (in hours).		
	0	2	4
E.H.A.	30	18	15
E.H.A. + toxin	30	4	1·8
E.H.A. + heated toxin (100° C. 30 minutes)	30	20	15

* Tests made with susceptible fowl cells.

The capacity of E.H.A. to agglutinate mouse cells appeared to be destroyed in parallel with its haemagglutinating activity for fowl cells. The results were somewhat obscured by the high susceptibility of mouse cells to haemolysis by the toxin.

The haemagglutinin of the Melbourne strain of influenza A virus, on the other hand, appeared to be quite resistant to the action of the toxin. Treatment of a 1: 10 dilution of allantoic fluid virus with toxin 1: 5,000 in the presence of 0·01 M calcium chloride resulted in no reduction of haemagglutinating activity even after incubation for 24 hours. It was shown in control tests that the presence of 1 :10 normal allantoic fluid did not interfere with the action of the toxin on V.H.A.

Action of Cobra Venom on Virus Haemagglutinins.

A different type of lecithinase, the so-called A type (Belfanti *et al*, 1936) which splits the unsaturated fatty acid from the lecithin molecule has been demonstrated in many snake venoms. It was decided, therefore, to test cobra venom for its ability to inactivate the vaccinia and ectromelia haemagglutinins.

TABLE 4.

Action of cobra venom on virus haemagglutinins.

Components of test mixture.	Haemagglutinin titre* after incubation for the following times (in hours).		
	0	1	2
V.H.A.	50	40	40
V.H.A. + cobra venom	40	4	< 2
V.H.A. + heated cobra venom (100° C. 30 minutes)	50	40	40
E.H.A.	30	—	18
E.H.A. + cobra venom	15	—	<2
E.H.A. + heated cobra venom (100° C. 30 minutes)	30	—	18

* Tests made with susceptible fowl cells.

The technique of testing was as follows: A 1: 10 dilution of a chorioallantoic emulsion was treated with cobra venom 1: 10,000 (in terms of dried material) at 37° C. in the presence of M/15 phosphate buffer pH 7·0. Haemagglutinin titrations were carried out in the presence of 1: 10 normal rabbit serum as this was found to reduce the haemolysis by mixtures containing cobra venom without affecting the titre of virus haemagglutination. The results given in Table 4

indicate that both V.H.A. and E.H.A. are rapidly destroyed by this concentration of cobra venom. Venom previously heated to 100° C. for 30 minutes in saline solution was inactive.

No evidence could be obtained of blockade of the action of cobra venom on V.H.A. by lecithin using 1: 50,000 cobra venom and 1: 500 lecithin.

Under the same conditions as used for cobra venom, hyaluronidase (1 p.c. suspension of rat testis) caused no destruction of V.H.A.

As with *Cl. welchii* toxin there was no inactivation of the haemagglutinin of the Melbourne strain of influenza A virus by cobra venom. A 1: 10 dilution of allantoic fluid virus in phosphate buffer was treated with 1: 10,000 cobra venom for 24 hours at 37° C. No inactivation took place apart from the slight fall in titre shown also by the control test without cobra venom. Normal allantoic fluid (1: 10) did not interfere with the action of cobra venom on V.H.A.

Semi-purified vaccinia haemagglutinin prepared by Mr. J. F. McCrea (unpublished data) by ammonium sulphate precipitation has been tested with *Cl. welchii* toxin and cobra venom. As with the crude preparations, the haemagglutinin was found to be destroyed by treatment with either enzyme preparation.

DISCUSSION.

It seems highly probable that the enzyme, lecithinase C (Macfarlane and Knight, 1941), is responsible for the inactivation of the vaccinia and ectromelia haemagglutinins by *Cl. welchii* toxin described in the foregoing experiments.

A number of different bacterial products, some of proven enzymic nature, have been identified in culture filtrates of *Cl. welchii* type A. According to Oakley (1943) the predominant toxin is the α toxin; θ toxin may also be present and possibly η toxin. Evans (1943) found hyaluronidase in filtrates of some type A strains. Other enzymes reported include collagenase (Macfarlane and MacLennan, 1945), and possibly lipolytic enzymes, in addition to lecithinase, and a desoxyribonuclease (Robb-Smith, 1945).

The sample of toxin used in these experiments had been precipitated with saturated ammonium sulphate. In addition to the α toxin or lecithinase, a small amount of proteinase was known to be present and presumably also some oxygen-inactivated θ toxin. However, the fact that the activity of the preparation against V.H.A. was so strikingly inhibited by citrate provides fairly conclusive evidence that this activity was due to lecithinase, requiring the presence of calcium or magnesium ions. The θ toxin is known to be active, at least in its haemolytic capacity, in the absence of calcium ions (van Heyningen, 1941).

The inactivity of the testicular extract indicates that hyaluronidase as well as a number of other common tissue enzymes are unable to bring about destruction of V.H.A.

Macfarlane and Knight (1941) have shown that the lecithinase of *Cl. welchii*, which they termed lecithinase C, splits lecithin alone and presumably also in certain protein complexes into a diglyceride and phosphocholine. It is unfortunate that a different terminology had previously been adopted by other workers (Contardi and Ercoli, 1933) in which lecithinase "C" was the enzyme hydrolyzing only the choline from the lecithin molecule and lecithinase "D", the glycerophosphatase corresponding to the type C of Macfarlane and Knight. In this discussion the terminology adopted is that of Macfarlane and Knight.

It appears from the small amount of work reported on the subject that the lecithinase C of *Cl. welchii* is highly specific in its activity. Macfarlane and Knight (1941) found that it did not hydrolyze simple phosphoric acid esters such as monophenyl-, diphenyl- or β glycero-phosphate; nor did it attack nucleic acid. A short note by Macfarlane (1942) states that it was inactive against purified brain cephalin but brought about slow hydrolysis of sphingomyelin. Taking this evidence at its face value, therefore, it would appear that the vaccinia and ectromelia haemagglutinins contain either lecithin or sphingomyelin.

The rapid destruction of the vaccinia and ectromelia haemagglutinins by highly diluted cobra venom is what would be expected if the effector group were

lecithin, but it is not easy to exclude the possibility that other enzymes than lecithinase were responsible. Although lecithinase A is the most widely recognized, other enzymes have been reported, including hyaluronidase (Duran-Reynals, 1939), choline-esterase and proteinase, as well as the still uncharacterized neurotoxin (Ghosh, De and Chowdhury, 1941). However, the fact that cobra venom is active to a dilution of 1 : 10,000 at least speaks for the activity of one of its major components, and taking into account the rest of the evidence that a phospholipid is concerned in the virus haemagglutination, the effect of cobra venom can be ascribed to lecithinase A with only formal reservations.

Lecithinase A acts on both cephalin and lecithin, attacking only the unsaturated fatty acid-glyceride linkage. It has no action on a synthetic lecithin containing only saturated fatty acids (Oppenheimer, 1943). Whether or not it can attack sphingomyelin, the other phospholipid in question, does not appear to have been recorded. As regards the fatty acid component of sphingomyelin, controversial views are held by different workers. According to Levene (1916) it contains no unsaturated fatty acids, but Merz (1930) postulated at least three forms which differ in the fatty acid component and one of which contains the unsaturated nervonic acid.

However, subject to reservations necessitated by the scanty information available on the enzymic breakdown of phospholipids other than lecithin, these experimental findings can be interpreted as indicating that the vaccinia and ectromelia virus haemagglutinins are substances containing lecithin and that enzymic breakdown of this component destroys the haemagglutinating properties.

No explanation in terms of the phospholipid component of the haemagglutinin is apparent for the one discrepancy in the properties of V.H.A. and E.H.A.—the fact that E.H.A. agglutinates mouse red cells and V.H.A. does not (Burnet and Stone, 1946). All three phospholipids—lecithin, cephalin and sphingomyelin—agglutinate mouse red cells.

It must be recognized that these conclusions apply only to the haemagglutinins of vaccinia and ectromelia viruses. The haemagglutination produced by viruses of the mumps-Newcastle disease-influenza group is a function of the virus particles themselves and involves a very different range of susceptible cells. The failure of either type of lecithinase to modify the agglutinating power of an influenza virus preparation is merely another indication of the sharp difference between the two groups.

SUMMARY.

Vaccinia and ectromelia virus haemagglutinins were inactiviated by treatment with *Cl. welchii* α toxin in the presence of calcium ions. The inactivation of vaccinia haemagglutinin was inhibited by citrate as well as by specific antitoxic serum. It was retarded by the presence of lecithin.

Cobra venom also produced rapid destruction of vaccinia and ectromelia haemagglutinins.

The haemagglutinin of the Melbourne strain of influenza A virus was not affected by *Cl. welchii* α toxin or cobra venom.

The action of these agents on the vaccinia and ectromelia haemagglutinins is attributed to lecithinases, type C in the case of *Cl. welchii* toxin, and type A in cobra venom.

It is concluded that these virus haemagglutinins contain a phospholipid, most probably lecithin, and that enzymic breakdown of this part of the complex destroys its haemagglutinating properties.

Acknowledgments. I am much indebted to Dr. F. M. Burnet for valuable advice throughout this work and to Dr. A. W. Turner for the supply of *Cl. welchii* toxin.

REFERENCES.

Belfanti, S., Contardi, A. and Ercoli, A. (1936): Ergebnisse Enzymforsch., 5, p. 213.
Burnet, F. M. and Stone, J. D. (1946): Austral. J. exp. Biol., 24, p. 1.
Contardi, A. and Ercoli, A. (1933): Biochem. Z., 261, p. 275.
Duran-Reynals, F. (1939): J. exp. Med., 69, p. 69.
Evans, D. G. (1943): J. Path. Bact., 55, p 427.
Ghosh, B. N., De, S. S. and Chowdhury, D. K. (1941): Ann. Biochem. and exp. Med. (Calcutta), 1, p. 31. Abstracted in Trop. Dis. Bull. 39, p. 718 (1942).
van Heyningen, W. E. (1941): Biochem. J., 35, p. 1246.
Levene, P. A. (1916): J. biol. Chem., 24, p. 69.
Macfarlane, M. G. (1942): Biochem. J., 36, part 1, iii (proceedings).
Macfarlane, M. G. and Knight, B. C. J. G. (1941): Biochem. J., 35, p. 884.
Macfarlane, R. G. and MacLennan, J. D. (1945): Lancet, 2, p. 328.
McClean, D., Rogers, H. J., Williams, B. W. and Hale, C. W. (1943): Lancet, 1, p. 355.
Merz, W. (1930): Z. physiol. Chem., 193, p. 59.
Oakley, C. L. (1943): Bull. Hyg., 18, p. 781.
Oakley, C. L. and Warrack, G. H. (1941): J. Path. Bact, 53, p 335.
Oppenheimer, C. (1943): "Die Fermente und ihre Wirkungen" Supplement Vol. 1, Edwards, Michigan.
Robb-Smith, A. H. T. (1945): Lancet, 2, p. 362.
Stone, J. D. (1946): Austral. J. exp. Biol., 24, p. 197.

LIPID HAEMAGGLUTININS

by JOYCE D. STONE[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 29th April, 1946.)

Extraction of a wide variety of tissues with organic solvents yields material which, when dispersed in saline, has active haemagglutinating properties. As described previously (Burnet and Stone, 1946) this mixture of lipids agglutinates essentially the same range of red cells as do the haemagglutinins of the vaccinia and ectromelia viruses. These cells include those from a proportion of fowls, some pigeons and with minor variations, all mice. Inhibition with dilute normal serum, however, distinguishes the lipid haemagglutinin from the antigenically-specific virus haemagglutinins. The present communication deals with investigations on the constitution of the lipid haemagglutinating system.

Little progress has been made in the field of isolation and identification of the haemagglutinin from tissue extracts, but we have found that agglutination of precisely the same type is given by certain purified lipids and more particularly by mixtures of lipids prepared in a manner allowing adequate dispersion.

MATERIALS AND METHODS.

Haemagglutinin titrations were made essentially as described previously (Burnet and Stone, 1946) using serial twofold dilutions and 2 p.c. fowl red cells. The starting dilution of the twofold series was judged so that a range of six tubes would cover the endpoint. Only fowls possessing fully "susceptible" cells, that is, with maximal sensitivity to lipid and vaccinia haemagglutinins, were used for this work. Tests were incubated at 37° C. and the preliminary shake after 15 minutes which is used for vaccinia titrations was omitted as maximal titres with lipid haemagglutinin, unlike the vaccinia haemagglutinin, are obtained at the first settling.

Titres are expressed as the reciprocal of the dilution (gm. per ml.) before the addition of the red cells, at which the "one plus" endpoint is shown. The lowest dilution used in the test was checked for failure to agglutinate insusceptible fowl cells. Except in the case of cephalin or where otherwise stated, the test substance was dissolved in alcohol in a concentration of 5–20 mg. per ml. and a 1: 100 dilution of the alcoholic solution made in saline. Cephalin preparations were suspended directly in saline from the dry state.

The preparations of the lipids were carried out by Mr. H. F. Holden of this Institute according to standard methods except as otherwise indicated in the text. The samples of lecithin, cardiolipin and sphingomyelin, prepared from beef heart, were supplied by Dr. M. C. Pangborn of the New York State Department of Health, Albany.

EXPERIMENTAL.

Tissue Extracts.

Attempts to isolate the active haemagglutinating principle from tissue extracts, both of chick embryo liver and baker's yeast have proved unsuccessful and have yielded little information on its character. It is readily soluble, at least in the crude state, in the common organic solvents: alcohol, acetone, chloroform, ether and petroleum ether. Difficulty was experienced, however, in obtaining exhaustive extraction of any one fraction, the haemagglutinin appearing in approximately equal concentration in a number of successive extracts.

For example, in one experiment in which the yeast lipid fraction, obtained by an initial extraction of dried yeast with hot alcohol, was extracted repeatedly with cold alcohol, there was practically no decline in the titres of a series of seven extracts. The titre of the first, in terms of the alcoholic solution, was 4×10^3 while that of the seventh was 2×10^3. In the light of results with cephalin fractions to be described later, it seems that this effect may have been due to the production of haemagglutinating material as an artefact during manipulations.

[1] This work was aided by a grant from the National Health and Medical Research Council.

The alternative approach to the general problem of the nature of the lipid haemagglutinin, namely, that of investigating the haemagglutinating properties of known lipids or mixtures of lipids likely to be present in these tissue extracts, has proved more fruitful.

Preliminary tests with a few common fatty acids, fats and oils, both saturated and unsaturated, indicated that these classes of compounds showed no appreciable activity. It was noticed, however, that although the titres were of a very low order, they were slightly higher with susceptible than with insusceptible cells. Two purified sterols, cholesterol and ergosterol, and a preparation of maize sterols (Eagle) were tested. Susceptible and insusceptible cells were agglutinated to approximately equal titres by each of these substances, the titres ranging from 5×10^3 to 20×10^3. Phospholipids on the other hand, with cephalin in particular, were found to possess relatively high haemagglutinating activity which, with the partial exception of cardiolipin, was entirely specific for the susceptible cells.

Haemagglutination by Individual Phospholipids.

Cephalin prepared from ox brain by the method of Folch and Schneider (1941) and stored for several years without special precautions against oxidation gave a haemagglutination titre of 1×10^6. During a fractionation of this material with chloroform-alcohol extraction followed by alcohol extraction, it appeared that the haemagglutinin was being continually produced, possibly as an oxidation product, and that this product was appreciably soluble in alcohol in contrast to the bulk of the cephalin.

Further evidence of this phenomenon was given by the following experiment in which three fractions, separated according to solubility in alcohol at room temperature, were allowed to stand on the bench in 2 p.c. aqueous suspension and titrated at intervals of several days. The two fractions insoluble in alcohol, one powdery, the other of a gummy consistency, showed considerable increase in titre, ten and four-fold, respectively, over a period of six days, while the corresponding alcohol soluble fraction remained unchanged. (See Table 1).

TABLE 1

Change in titre of cephalin fractions on standing in aqueous suspension at room temperature.

Fraction.	Time in days.			
	0	2	6	14
Alcohol insoluble, powdery	$1{\cdot}4 \times 10^6$	$4{\cdot}7 \times 10^6$	$1{\cdot}8 \times 10^7$	$1{\cdot}4 \times 10^6$
" " gummy	$1{\cdot}4 \times 10^6$	$2{\cdot}4 \times 10^6$	$5{\cdot}3 \times 10^6$	$4{\cdot}7 \times 10^6$
Alcohol soluble	$2{\cdot}4 \times 10^6$	not tested	2×10^6	$1{\cdot}5 \times 10^6$

In order to determine the activity of newly prepared material a fresh preparation of cephalin was undertaken using the recent method of Folch (1942) for sheep brain. Haemagglutinin titrations were carried out on all fractions obtained during the course of the preparation.

The preliminary acetone and alcohol extracts which are discarded from the preparation gave titres in the range 2×10^4 to 6×10^5. The cephalin fraction, after extraction in petroleum ether followed by solution in ethyl ether and precipitation with alcohol, had a titre of $4{\cdot}4 \times 10^4$, while the fraction not precipitated by alcohol at this stage had a titre of $1{\cdot}7 \times 10^5$. A second precipitation with alcohol did not significantly reduce the titre of the final cephalin fraction.

A portion of this cephalin was allowed to stand at room temperature freely exposed to air and diffuse sunlight while the remainder was stored at 0° C. *in vacuo*. After 7 days the exposed sample, which had turned from white to a brownish yellow colour, gave a titre of $6{\cdot}4 \times 10^5$ while the activity of the material stored *in vacuo* had not changed ($4{\cdot}5 \times 10^4$). After a further 6 weeks' storage both samples showed the same titres as after 7 days. When making the initial aqueous suspensions of these materials it was noticed that the exposed sample dispersed to form a uniform and stable suspension much more readily than the vacuum-stored portion which tended to remain in larger aggregates.

Lecithin was prepared from egg yolk according to the method of Levene and Rolf (1927). The final product when suspended in saline from the dry state gave no haemagglutination at dilutions of 1 in 4×10^2 or greater. After exposure to air for 5 days no change was detected, but after 14 days weak agglutination to a titre of 1×10^4 was observed, the control material stored *in vacuo* over this period remaining negative. A third test after 22 days' exposure revealed no further increase in titre.

When the vacuum-stored material was dissolved in alcohol and saline dilutions prepared from this, some agglutination (titre $1{\cdot}2 \times 10^3$) occurred. This titre increased on a second reading to that given by the exposed material tested in the same manner ($1{\cdot}5 \times 10^4$). The greater activity of suspensions prepared from fresh lecithin via solution in alcohol may be due either to oxidation of the lecithin in alcoholic solution or to the better dispersion of the suspension in saline.

TABLE 2.

Haemagglutination by mixtures of heart lecithin and cholesterol in different ratios.

Cholesterol dilutions.	Lecithin dilutions. 6,400	1,600	400
1,600	1/4*	1/1	4/1
	1,000†	2,800	800
	$(1{\cdot}3 \times 10^7)$‡	$(9{\cdot}0 \times 10^6)$	$(6{\cdot}4 \times 10^5)$
400	1/16	1/4	1/1
	180	3,200	10,000
	$(2{\cdot}3 \times 10^6)$	$(1{\cdot}0 \times 10^7)$	$(8{\cdot}0 \times 10^6)$
100	1/64	1/16	1/4
	<100	800	4,800
	$(<1{\cdot}3 \times 10^6)$	$(2{\cdot}6 \times 10^6)$	$(3{\cdot}8 \times 10^6)$

* Ratio of lecithin to cholesterol.
† Titre in terms of alcoholic mixture.
‡ Titre in terms of lecithin.

A preparation of lecithin from beef heart supplied by Dr. Pangborn in the form of an alcoholic solution had a titre of $3{\cdot}5 \times 10^4$.

Lysocephalin and Lysocithin. Only crude preparations of lysocephalin and lysocithin (contaminated with lysocephalin) which had been prepared by the action of cobra venom on egg yolk (Feldberg, Holden and Kellaway, 1938) were available for testing. These were haemolytic to titres of 2×10^4 and 4×10^4 respectively. No haemagglutination was observed beyond these dilutions and there was no differential haemolytic effect on susceptible and insusceptible cells.

Sphingomyelin. A crude sample of sphingomyelin was prepared from ox brain as follows. The brain was dried *in vacuo* and extracted thoroughly with acetone. The insoluble residue was extracted with ethyl alcohol and the united extracts evaporated to dryness *in vacuo*. After extraction with ether several times the residue was dissolved in warm dry pyridine and allowed to deposit the crude sphingomyelin slowly at 0° C. The deposit was centrifuged off and dried *in vacuo*.

This material gave weak, atypical agglutination, the pattern slipping before sedimentation was complete. By taking a reading about 15 minutes after setting up the test a titre of $2{\cdot}4 \times 10^5$ was obtained.

Stable agglutination to approximately the same level, $2{\cdot}5 \times 10^5$, was given by a specimen of purified sphingomyelin prepared from beef heart by Dr. Pangborn.

Cardiolipin. This phospholipid isolated from beef heart extracts by Pangborn (1942) appears to be an essential constituent of the Wassermann antigen (Pangborn, 1942; Maltaner and Maltaner, 1945). Although not yet fully identified, it is thought to be an acidic, non-nitrogenous phospholipid present in the tissues as the sodium or potassium salt (Pangborn, 1944).

Cardiolipin gave somewhat variable results in the haemagglutination test. A titre of approximately 3×10^5 was obtained with susceptible fowl cells increasing to 1×10^6 when the test was shaken and allowed to settle a second time. Insusceptible fowl cells were also agglutinated but to a lower titre (8×10^4) and the agglutination was of a coarse rather atypical type and decreased considerably on the second reading.

From this limited survey of the haemagglutinating properties of individual lipids it did not appear that any of the substances tested corresponded with the haemagglutinating agent present in tissue extracts. For this reason, and also in view of the findings of Pangborn and collaborators on the composite nature of the Wassermann antigen system, it seemed desirable to test out various mixtures of known lipids as haemagglutinating agents.

Haemagglutination of Mixtures of Lipids.

In preliminary experiments in which mixtures of lecithin and cholesterol were made in a saline medium, no increased haemagglutination occurred. However, when the lipids were first mixed in alcoholic solution and then the mixture diluted in saline, titres considerably greater than those given by the separate components were observed. The general technique, therefore, was to prepare a mixture of equal parts of the appropriate alcoholic solutions. This was then diluted serially in saline making an initial 1: 100 dilution by dropping 0·05 ml. into 5 ml. of saline with vigorous shaking.

TABLE 3.

Haemagglutination by mixtures of egg lecithin and cholesterol in different ratios.

Cholesterol dilutions.	Lecithin dilutions. 1,600	800	400	200
800	1/2* 3,000† (9·6 × 10⁶)‡	1/1 4,000 (6·4 × 10⁶)	2/1 1,200 (9·6 × 10⁵)	4/1 <100 (<4 × 10⁴)
400	1/4 300 (9·6 × 10⁵)	1/2 6,000 (9·6 × 10⁶)	1/1 8,000 (6·4 × 10⁶)	2/1 2,400 (9·6 × 10⁵)
200	1/8 <100 (<3·2 × 10⁵)	1/4 1,000 (1·6 × 10⁶)	1/2 3,500 (2·8 × 10⁶)	1/1 16,000 (6·4 × 10⁶)
100	1/16 <100 (<3·2 × 10⁵)	1/8 800 (1·3 × 10⁶)	1/4 3,000 (2·4 × 10⁶)	1/2 2,000 (8·0 × 10⁶)

* Ratio of lecithin to cholesterol.
† Titre in terms of alcoholic mixture.
‡ Titre in terms of lecithin.

During the course of these experiments it was noticed that dilutions of cholesterol-containing mixtures made in calcium saline (calcium chloride 0·024 p.c., sodium chloride, 0·9 p.c.), our usual diluent for haemagglutination tests, flocculated more rapidly and markedly than did the corresponding dilutions in normal saline. This flocculation appeared to be accompanied by decreased haemagglutinating activity so that for this work, normal saline has been substituted as the diluting fluid. Comparative tests revealed no difference in the titres of tissue extracts in the two diluents.

The "specific" nature of the haemagglutination of all mixtures was checked by the inability to agglutinate insusceptible cells. The haemagglutinin titres reported below are for susceptible cells only.

TABLE 4.

Haemagglutination by mixtures of heart lecithin and cardiolipin in different ratios.

Cardiolipin dilutions.	Lecithin dilutions. 6,400	1,600	400
6,400	1/1* 130† (1·7 × 10⁶)‡ (1·7 × 10⁶)‖	4/1 400 (1·3 × 10⁶) (5·1 × 10⁶)	16/1 150 (1·2 × 10⁵) (1·9 × 10⁶)
1,600	1/4 140 (1·8 × 10⁶) (4·5 × 10⁵)	1/1 3,200 (1·0 × 10⁷) (1·0 × 10⁷)	4/1 4,000 (3·2 × 10⁶) (1·3 × 10⁷)
400	1/16 <400 (<5·1 × 10⁶) (<3·2 × 10⁵)	1/4 <800 (<2·6 ×10⁶) (<6·4 × 10⁵)	1/1 8,000 (6·4 × 10⁶) (6·4 × 10⁶)

* Ratio of lecithin to cardiolipin.
† Titre in terms of alcoholic mixture.
‡ Titre in terms of lecithin.
‖ Titre in terms of cardiolipin.

Mixtures of cardiolipin, cholesterol and lecithin. Combinations of these three compounds which comprise Pangborn's Wassermann antigen system, were studied in some detail and the optimum ratio for each of the three pairs was determined. For this purpose mixtures of a series of alcoholic dilutions of each component were made and titrated as described below. As these

experiments were designed primarily to show the increase in titre obtained with mixtures, all the reagents were used at dilutions beyond those capable of producing agglutination when acting alone. Controls of each reagent at the lowest dilution used were included in the tests but are not shown in the Tables.

In the case of lecithin (heart)-cholesterol mixtures, maximal haemagglutination occurred with ratios of lecithin to cholesterol in the range 1: 1 to 1: 4, the titres in terms of lecithin being approximately 1×10^7 (Table 2). Alteration of the ratio to 4: 1 or 1: 16 was accompanied by a significant decrease in the haemagglutination titre. Similar results were obtained using egg lecithin (Table 3) and it is interesting to note that both vacuum-stored lecithin, showing practically no activity *per se*, as well as the exposed lecithin, appeared equally active in the presence of cholesterol.

Lecithin-cardiolipin mixtures were also highly active, optimum titres (1×10^7) being obtained with the ratios, lecithin to cardiolipin, 4: 1 to 1: 1 (Table 4).

The third combination, cardiolipin and cholesterol, resulted in relatively slight increase in activity. The titres, approximately 2×10^6, obtained with the first readings of the mixtures were only slightly above those given at the second readings of cardiolipin alone. Little difference was found over a wide range of ratios of cardiolipin to cholesterol from 10: 1 to 1: 50 although with ratios greater than unity the agglutination was of weaker character.

A mixture of all three compounds in the ratio cardiolipin-lecithin-cholesterol, 1: 4: 20, showed no significant increase in activity over that of either of the pairs, cardiolipin and lecithin or cholesterol and lecithin. Table 5 shows the results of one experiment in which all possible combinations of the three compounds were titrated in parallel.

TABLE 5.

Haemagglutination with various combinations of cardiolipin, lecithin and cholesterol.

Constituents of mixture.*	Haemagglutination of susceptible fowl cells. Dilutions of mixture.									Reaction with insusceptible cells.
	100	200	400	800	1,000	2,000	4,000	8,000	Titre.	100
Cardiolipin	±	−	−	−	−	−	−	−	<100	−
Lecithin	++	++	+$^+$	−	−	−	−	−	500	−
Cholesterol	−	−	−	−	−	−	−	−	<100	−
Cardiolipin and cholesterol	±	−	−	−	−	−	−	−	<100	−
Cardiolipin and lecithin	++	++	++	++	++	++	+$^+$	−	5,000	−
Lecithin and cholesterol	++	++	++	++	++	++	±±	+	8,000	−
Cardiolipin, lecithin and cholesterol	++	++	++	++	++	++	±±	+	8,000	−

* The mixtures were made with equal volumes of alcoholic solutions of cardiolipin 1: 2,000, lecithin 1: 400 and cholesterol 1: 100 as indicated. All were brought to three volumes with the appropriate amount of alcohol.

++ Complete haemagglutination.

− No agglutination.

Other lipids combined with cholesterol or lecithin. A number of other compounds have been tested for activity when substituted for one or other component in the lecithin-cholesterol system. Those compounds tested in combination with cholesterol will be considered first.

Mixtures containing equal parts of test substance and cholesterol were prepared from alcoholic solutions of equal concentration, in most cases 1: 100. These were then titrated starting with an initial 1: 100 dilution in saline in the usual manner. The titres, expressed in terms of the original test substances, are listed in Table 6 together with control titres obtained when the test substance was mixed with alcohol alone. Lecithin and cardiolipin are included in the series for purposes of comparison.

As will be seen from the Table, sphingomyelin gave increased agglutination in the presence of cholesterol, the titre being $3 \cdot 2 \times 10^6$ as compared with $1 \cdot 6 \times 10^5$ in the control without cholesterol. Lysocephalin and a mixed preparation of lysocephalin and lysocithin also appeared satisfactory substitutes for lecithin, although in view of the impure nature of the preparations these results must be taken with reserve. The alcohol soluble fraction of oxidized cephalin was not activated by the addition of 1 p.c. cholesterol nor was a non-cholesterolized Wassermann antigen preparation (Eagle).

An adequate test of fresh cephalin and cholesterol could not be made as no mutual solvent miscible with water was available. When mixtures were made in saline there was no enhancement of the original activity.

TABLE 6.

Haemagglutination by mixtures containing cholesterol.

Test substance.	Titre in presence of cholesterol.	Titre without cholesterol.
Lecithin (heart)	8×10^6	3×10^4
Lecithin (egg)	$6{\cdot}4 \times 10^6$	$1{\cdot}2 \times 10^4$
Cardiolipin	$1{\cdot}6 \times 10^6$	3×10^5*
Sphingomyelin (heart)	$3{\cdot}2 \times 10^6$	$1{\cdot}6 \times 10^5$
Lysocephalin (crude)	2×10^6	$<2 \times 10^4$
Lysocithin (crude)	$2{\cdot}4 \times 10^6$	$<4 \times 10^4$
Eagle's heart extract antigen	4×10^3	4×10^3
Alcohol soluble fraction of oxidized cephalin	4×10^6	$3{\cdot}2 \times 10^6$
Oleic acid	7×10^4†	$3{\cdot}6 \times 10^4$
Palmitic acid	4×10^5†	$<2 \times 10^4$
Stearic acid	2×10^5†	$<2 \times 10^4$
Glycol distearate	3×10^5	$<2 \times 10^4$
Sodium tauroglycocholate (B.D.H.)	$4{\cdot}8 \times 10^5$	$<2 \times 10^4$

* Increasing to 1×10^6 on second reading.
† Mixture agglutinates insusceptible cells also (see text).

Sodium tauroglycocholate (British Drug Houses) and glycol distearate showed weak activity in the presence of cholesterol. Mixtures of oleic, palmitic or stearic acid and cholesterol gave low titre agglutination of both susceptible and insusceptible cells, although in the case of palmitic and stearic acids the titres were four to eightfold higher with the susceptible cells.

A second series of tests has been carried out with various substances in combination with lecithin. As before mixtures were made with equal concentrations of the two reagents in alcoholic solution. Of a number of compounds chemically related to cholesterol, only cholesterol acetate, ergosterol and a preparation of maize sterols gave active mixtures with lecithin, while hexoestrol, stilboestrol, desoxycorticosterone acetate, cholic acid and methyl cholanthrene were inactive (Table 7). In addition to those listed in the table other ring compounds which have been tested and found negative include pyrene, phenanthrene, phenanthraquinone, anthraquinone, naphthalene and o–diphenyl benzene. A mixture of anthracene and lecithin showed slight activity, 5×10^5. The three fatty acids, oleic, palmitic and stearic, also glycol distearate and sodium tauroglycocholate gave practically inactive mixtures.

It is interesting to note that the addition of lecithin to three preparations, sphingomyelin, Wassermann antigen (beef heart extract) and the alcohol soluble fraction of exposed cephalin not only caused no activation but was accompanied by a definite depression of haemagglutination (Table 7).

TABLE 7.

Haemagglutination by mixtures containing lecithin.

Test substance.	Titre in presence of lecithin.	Titre without lecithin.
Cholesterol	7×10^6	$<1 \times 10^5$
Cholesterol acetate	$1{\cdot}2 \times 10^6$	"
Ergosterol	$1{\cdot}6 \times 10^6$	"
Maize sterols	$4{\cdot}8 \times 10^6$	"
Hexoestrol	$<1 \times 10^5$	"
Stilboestrol	$<1 \times 10^5$	"
Desoxycorticosterone acetate	$<1 \times 10^5$	"
Cholic acid	$<1 \times 10^5$	"
Methyl cholanthrene	$<1 \times 10^5$	"
Sphingomyelin	7×10^4	$1{\cdot}6 \times 10^5$
Eagle's heart extract antigen	6×10^2	$2{\cdot}5 \times 10^4$
Alcohol soluble fraction of oxidized cephalin	4×10^5	$3{\cdot}2 \times 10^6$

Summarizing, therefore, it may be stated that a mixture of lecithin and cholesterol exhibits high haemagglutinating activity and that the lecithin is replaceable by sphingomyelin and the cholesterol by (a) ergosterol, maize sterols or (b) cardiolipin without loss of this activity.

Taking a lecithin-cholesterol mixture as the type case, the general properties of these lipid mixtures have been checked and found in agreement with those of the tissue lipid haemagglutinin. Firstly, the agglutination titre was considerably higher at 37° C. than in the refrigerator. Secondly, the activity was readily inhibited by normal serum, eight agglutinating doses being inactivated by two rabbit sera at all dilutions up to 640 and 800 respectively. Thirdly, the mixture agglutinated susceptible pigeon cells and mouse cells.

TABLE 8.

Depressant effect of lecithin on haemagglutination of insusceptible cells.

Components of mixture.	Dilutions of mixture.						Titre.
	10	20	40	80	160	320	
Cardiolipin 1:100 with alcohol	.	++	++	++	+⁺	tr.	200
Cardiolipin 1:100 with lecithin 1:400	.	+⁺	+⁺	+⁺	±	tr.	100
Cardiolipin 1:100 with lecithin 1:100	—	—	—	—	—	—	<10
Cholesterol 1:100 with alcohol	++	±±	tr.	—	—	—	30
Cholesterol 1:100 with lecithin 1:400	tr.	—	—	—	—	—	<10
Cholesterol 1:100 with lecithin 1:100	—	—	—	—	—	—	<10

It should be emphasized that in the above tests, except where otherwise stated, the increased activity in mixed preparations of lipids was demonstrable only with susceptible fowl cells. The weak haemagglutination of insusceptible cells which is shown by some compounds such as cholesterol and cardiolipin is actually depressed by the addition of lecithin. This effect was demonstrated by the experiment reported in Table 8 in which cholesterol and cardiolipin with and without the addition of lecithin were titrated with insusceptible fowl cells.

DISCUSSION.

The fact that alcoholic mixtures of purified lipids such as lecithin and cholesterol are active haemagglutinating agents when diluted in saline offers a reasonable explanation of the activity of our tissue extracts. In any such extract there will normally be a mixture of phospholipids and sterols and the main haemagglutinating activity can be accounted for by lecithin-cholesterol combinations in the animal tissue extracts with ergosterol replacing the cholesterol in the yeast extracts.

It is essential for the high activity of such mixtures that the components be mixed first in a state of true solution, as in alcohol, before being dispersed in saline. Of interest in this connection is the hypothesis of Eagle (1937) on the sensitizing action of cholesterol on the Wassermann antigen, namely that the microcrystals of cholesterol provide nuclei on which the antigen is adsorbed. One might postulate such a state of affairs with our lipid mixtures, the phospholipid being distributed on the surface of cholesterol aggregates and coincidently serving to stabilize the cholesterol suspension in the aqueous medium. This dispersing action of lecithin is well known and was in fact apparent in our tests with mixtures of lecithin and other compounds. The addition of lecithin to hydrophobic compounds such as cholesterol was regularly accompanied by a decreased turbidity on subsequent dilution in saline as compared with the dilutions of the compounds alone.

Whether the two components of the mixtures have specific configurative parts to play in the haemagglutination reaction, or whether one merely serves as an inactive nucleus for the suitable distribution of the other component, cannot be decided upon at this juncture.

In terms of the phospholipid-sterol hypothesis, the position with cephalin is not easily interpreted. Owing to insolubility in alcohol, cephalin could not be adequately tested with the addition of cholesterol. However, after exposure of cephalin to oxidation or other deteriorative process, a system with high activity is in some way developed. This may be due solely to the activity of the new product or possibly, in line with the above hypothesis, to the more effective distribution of the cephalin itself in the presence of this second product. Cholesterol does not seem to play a part in the system or else its effect is masked by the already existing high activity, as no cholesterol could be detected in the alcohol soluble fractions of the oxidized cephalin by a test with sulphuric acid and acetic anhydride, nor did any increased activity result from the addition of cholesterol to these fractions.

The activity of beef heart extracts prepared as Wassermann antigen is also not readily explained in terms of any simple system. These extracts are active haemagglutinating agents although very little cholesterol can be present. Furthermore, the addition of cholesterol to the extracts does not result in increased haemagglutination. This activity may be due to the same components as are responsible for the haemagglutination of cephalin preparations. Alternatively, cardiolipin which is more active in combination with lecithin than with cholesterol, may be responsible.

The depressant effect of lecithin on the agglutination of insusceptible cells by cholesterol and cardiolipin is of particular interest, as it would provide an explanation of the complete inactivity of tissue extracts against insusceptible cells, although they contain such compounds as cholesterol and cardiolipin.

There is an interesting analogy between the interpretation we have placed on lipid haemagglutination and the current hypotheses on the nature of the Wassermann antigen. All the reagents prepared for complement fixation or precipitation tests in syphilis that we have examined including Bordet, Eagle and Kahn antigens, as well as Pangborn's three component antigen of purified cardiolipin, lecithin and cholesterol are in fact active haemagglutinins. The two activities both appear to be based on suitably dispersed lipid combinations but the Wassermann reactivity is much more restricted than the haemagglutinating action which is shown equally well by some mixtures which are quite inert in serological tests.

Lipid haemagglutination is of biological interest, chiefly in its bearing on the problem of the nature of the specific haemagglutinins of vaccinia and ectromelia viruses. The almost exact correspondence in the range of cells agglutinated by the two agents, a range which differs strikingly from that covered by any other type of virus or serological agglutinin, makes it almost certain that the active molecular configurations are similar in both groups of agents. Our provisional picture of the vaccinia haemagglutinin is as a relatively well-defined and stable complex of a virus antigen and a phospholipid.

From the practical standpoint the existence of nonspecific lipid material with the same type of agglutinating activity must always be borne in mind during work with the vaccinia group of haemagglutinins and in any search for hitherto unrecognized agents of similar type. The most satisfactory means of differentiation is based on the resistance of the virus haemagglutinins and the susceptibilty of the lipid haemagglutinins to inactivation by dilute normal serum.

SUMMARY.

Lipid extracts from a variety of normal tissues agglutinate the same range of red blood cells as vaccinia and ectromelia virus haemagglutinins. In an attempt to identify the agent responsible in such extracts a number of purified lipids have been tested for haemagglutinating activity both alone and in mixture.

Activity of individual lipids was confined to the phospholipid group. There was considerable variation in the titres of the four representatives, cephalin, lecithin, sphingomyelin and cardiolipin. Phospholipids gave increased haemagglutination titres when dispersed in saline from alcoholic solutions containing cholesterol. Ergosterol and a preparation of maize sterols were satisfactory substitutes for cholesterol in this combination.

The main haemagglutinating activity of the lipid fraction of animal tissues is attributed to combinations of lecithin and cholesterol, with ergosterol replacing the cholesterol in the case of yeast extracts.

Acknowledgments. Mr. H. F. Holden has been responsible for all preparations of lipid material involved in this investigation. For this and for much helpful advice I wish to extend my thanks. I am also greatly indebted to Dr. F. M. Burnet who has given valuable criticism and guidance throughout the work. Dr. M. C. Pangborn kindly supplied samples of cardiolipin, lecithin and sphingomyelin.

REFERENCES.

Burnet, F. M. and Stone, J. D. (1946): Austral. J. exp. Biol., 24, p. 1,
Eagle, H. (1937): "The Laboratory Diagnosis of Syphilis", Kimpton, London.
Feldberg, W., Holden, H. F. and Kellaway, C. H. (1938): J. Physiol., 94, p. 232.
Folch, J. (1942): J. biol. Chem., 146, p. 35.
Folch, J. and Schneider, H. A. (1941): *Ibid.*, 137, p. 51.
Levene, P. A. and Rolf, I. P. (1927): *Ibid.*, 72, p. 587.
Maltaner, R. and Maltaner, F. (1945): J. Immunol., 51, p. 195.
Pangborn, M. C. (1942): J. biol. Chem., 143, p. 247.
Pangborn, M. C. (1944): *Ibid.*, 153, p. 343.

SEROLOGICAL RESPONSE TO INFLUENZA B INFECTION IN HUMAN BEINGS

DIFFERENTIATION OF SPECIFIC AND NONSPECIFIC TYPE REACTIONS

by F. M. BURNET AND JOYCE D. STONE[1]

(With the technical assistance of Margaret Kerr).

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 17th May, 1946.)

In 1935 Andrewes, Laidlaw and Smith showed that in most adult human sera antibodies were present against both influenza (A) and swine influenza viruses. In children under 12, however, when influenza antibodies were present they were active only against the human strain (W.S.), not against swine influenza virus. Their first interpretation that the swine type antibodies represented a residuum of antibody resulting from infection with pandemic type virus has been disproved by the finding reported by Stuart Harris *et al.* (1938) that adults suffering their first infection with influenza produced both types of antibody.

An extensive study of human antisera made by Burnet and Lush (1937) with the choriollantoic technique allowed a fairly clear demonstration that in children before adolescence the antibodies present were relatively specific, acting to full titre only against Melbourne type virus and showing low values with W.S. and Swine 15 viruses. In adults the levels against the three strains were usually commensurate.

Rickard, Thigpen and Adams (1945) have recently confirmed this work by showing that young children suffering their first infection with influenza A give antibody responses much higher for the homologous strain than for W.S. or swine influenza viruses. Antibody against the latter strains also falls proportionately faster than the homologous strain so that the antibody pattern of the serum becomes progressively more specific.

It has been found by Gordon (1942) and Burnet, Beveridge and Bull (1944) that there are considerable serological differences amongst influenza B strains, and it is reasonable to suppose that extensive study of B strains isolated in various times and countries might show the same wide range of minor serological differences as is known to exist amongst A strains. In view of the possible importance of such differences in the preparation of effective immunizing agents an attempt was made to assess the type of immunological response to infection in a presumably homogeneous B epidemic, using both a homologous strain and the standard LEE strain in the tests.

MATERIALS AND METHODS.

The sera were obtained during the investigation of the influenza B epidemic of October-November, 1945 (Burnet, Stone and Anderson, 1946). They were heated to 56° C. for 30 minutes and stored in a refrigerator in tightly corked tubes with the addition of 1: 10,000 methiolate. The viruses used were LEE, received as mouse adapted material from Dr. Francis in 1941, BON (Victoria, 1943), HAW (Pacific Islands, 1945) and MIL–B, ELI, HUT and GUN (Victoria, 1945), all these being isolated and maintained by chick embryo methods.

Antihaemagglutinin titrations were made initially by the standard method used in earlier investigations using 2 p.c. fowl cells and five agglutinating doses of virus. A more satisfactory method designed to overcome the difficulty of the nonspecific inhibitory titres shown by certain

[1] This work was aided by a grant from the National Health and Medical Research Council.

normal sera was used later. This technique which will be described elsewhere is modified from that of Salk (1944), the only essential difference being that the fowl cells are made from specially selected birds. There are sharp differences between fowl cells from different birds in regard to the inhibitory titre given by a serum known to contain no influenza antibody and tested throughout with the same virus dilution. Only cells from about 15 p.c. of fowls are capable of giving titres below 10 with the two B strains used in the present work and all titrations by the second method were made with such cells. The cells were added as a 0·5 p.c. suspension giving a final concentration of 0·17 p.c. The virus was diluted to give one standard agglutinating dose with 2 p.c. cells (equivalent to 4–5 agglutinating doses with 0·5 p.c. cells). The endpoint was a standard degree of partial agglutination read after the cells had been allowed to settle, then reshaken and allowed to settle again at room temperature throughout. *Allantoic titrations* were made according to the technique described by Burnet (1943) the virus strains LEE and MIL–B both well adapted to growth in the allantoic cavity, being used. A single stock of each virus was maintained in the form of amniotic fluid plus 30 p.c. glycerol. This was stored in a freezing chamber at —10° C. and showed very little drop in titre over the period of the tests. Details as to the method of expressing the results are more conveniently given later.

Specific and Nonspecific Antihaemagglutinin Response.

In studying the serological reactions of persons (largely adolescents) infected with influenza B during the Victorian epidemic of October-November, 1945, we carried out haemagglutinin tests using both a standard strain LEE–B and two strains isolated during the epidemic, GUN–B and MIL–B. In the first large group of cases which was studied, adolescent boys from School 3, it was noticeable that the titres of the convalescent samples of serum fell into two patterns. In one the titres against the homologous strain were several times higher than those against LEE. In the other type the titres with LEE were equal or greater than those with the homologous strain.

Table 1 shows the titres obtained in this group, the first set being obtained by the standard technique with 2 p.c. cells and the second using the modified Salk technique with 0·5 p.c. cells from specially selected fowls. It will be seen that with minor discrepancies the division of the patients into two groups is clearly shown.

TABLE 1.

Antibody titres of convalescent sera from a group of patients 14–16 years of age.

Patient.	GUN.	LEE.	MIL.	LEE.	Patient.	GUN.	LEE.	MIL.	LEE.
MER	100	15	250	60	GOD	440	300	480	1,000
MIL	560	120	800	240	LET	160	280	560	1,000
HEY	160	70	480	120	THO	380	320	480	600
MILS	50	40	60	30	ROS	300	320	640	1,280
CLA	120	20	260	70					
STO	140	35	600	200					
WIL	120	35	25	30					
ALL	55	15	14	30					
WIS	220	30	...	...					

In the course of our serological work on this epidemic 37 pairs of acute and convalescent sera were found to show a significant rise in influenza B antibody between first and second bleedings. The distribution of specific responses (with MIL or GUN showing a higher titre than LEE) and nonspecific responses (LEE higher than MIL or GUN) is shown in Table 2. The concentration of specific responses in the adolescent group and of nonspecific ones amongst adults is probably significant. It is possible that a more clear-cut difference would have been oberved if a wider range of ages had been available. There were very few of our subjects outside the limits of 13 to 25 years.

Relationship amongst Influenza B Strains when Tested with Immune Sera.

In the specific group of sera shown in Table 1 the sharp difference in titres obtained against the homologous strain MIL–B and the standard LEE strain indicated the possibility of using human sera for subtype differentiation within the group of influenza B viruses. Reciprocal haemagglutination tests were, therefore, made using as far as possible strictly homologous viruses and sera. The specific and nonspecific sera, CLA II and ROS II respectively, were from the outbreak in School 3 from which virus MIL–B was isolated. Sera and viruses ELI and HUT were from two individuals infected at the same period but in quite different communities. The other virus strains used were from unrelated epidemics, viz. the classical LEE strain isolated in America in 1940, BON isolated in Victoria in 1943 and a strain HAW received from Major Millstone which had been isolated in the Pacific area in June, 1945.

To compare the strains the crude titres were adjusted in a similar fashion to that described by Friedewald (1944) in order to allow for differing avidities and varying antibody content of the sera used. The procedure was first to reduce all titres obtained with each serum to the percentage of that given by virus MIL. This was designed to eliminate differences corresponding to the absolute amount of antibody present in the sera. It was then assumed that serum ROS which on inspection appeared to be the most widely active of those available had an equal activity against all viruses in the group. The second set of titres was then adjusted so that for each virus the titre given by ROS was taken as 100 and the corresponding titres with other sera expressed as the appropriate percentage of that given with ROS. In this way varying avidities of the different virus strains is allowed for. Fig. 1 shows the results after the final adjustment. The results with serum HUT are unreliable owing to the low absolute titre of the serum.

Fig. 1 indicates clearly the sharp differentiation of LEE from the other strains and suggests that the latter including HAW and BON fall into a closely related group. Using the ordinary conventions of immunological reasoning the figure suggests that three activities of the sera can be distinguished.

1. Neutralization of LEE due to factor A which is present in ROS, ELI and HUT sera but absent in CLA serum.
2. Full neutralization of all other viruses than LEE: factor B present only in ROS serum.
3. Neutralization governed by a diminishing susceptibility of the viruses when arranged in the order shown and corresponding to an antigenic component present or available in them in diminishing amount: factor C present in HUT, ELI, CLA and presumably in ROS sera.

The factors postulated must not of course be interpreted as necessarily well defined antigenic groups and they do not perhaps signify much more than that human beings differ greatly in the details of their immunological response to an apparently uniform stimulus. The results do, however, establish an important difference between LEE in its present state and the other more recently isolated B strains. Since the findings are with human sera modified by natural infection they suggest strongly that LEE alone should not be used for the production of vaccines for immunization against influenza B.

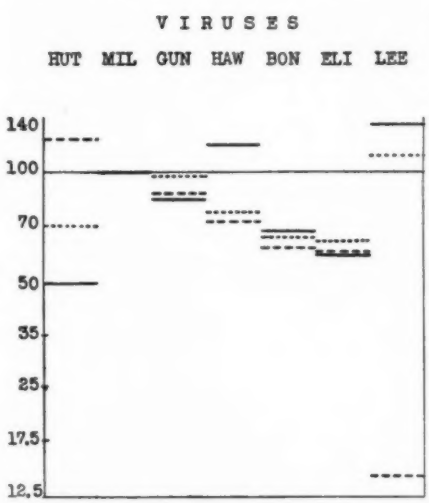


Fig. 1. Corrected titres of four human immune sera against seven influenza B viruses.

Thin continuous line, Serum ROS.
Heavy continuous line, Serum HUT.
Broken line, Serum CLA.
Dotted line, Serum MIL.
For details of construction see text.

Allantoic Neutralization Reactions.

We have been unable to adapt any of the B strains other than LEE to mouse passage so that *in vivo* neutralization tests must be done by the allantoic method described independently by Hirst (1942) and ourselves (Burnet and Beveridge, 1943).

The method used was to inoculate suitable mixtures of serum and virus after contact for one hour at 4° C. into the allantoic cavity of 11-day chick embryos. After three days' incubation at 35° C. a small amount of allantoic fluid from each egg was tested for haemagglutination. With sera containing little or no antibody, tests were made with mixtures of undiluted serum plus serial tenfold dilutions of virus. With active sera virus diluted 1: 100 was mixed with equal volumes of serum diluted 1, 3, 10, 30 and 100 fold. Six eggs were used in each mixture and the endpoint 50 p.c. infection determined by Reed and Muench's method.

The whole series reported was done using stocks of amniotic fluid virus preserved with 30 p.c. glycerol and stored at — 10° C. No lower dilutions than 1 :100 were used. The activity of a serum is expressed as the serum dilution which mixed with 1 :100 virus representing 10^4 infective doses will produce 50 p.c. of infections in three days Allowing for the twofold dilutions of virus in all mixtures the standard virus will have a titre of $10^{6\cdot3}$.

With sera which undiluted failed to neutralize virus diluted 1: 100, the titre was expressed according to a convention based on the fact that the neutralization curve, when logarithmic concentrations of virus and antibody are used as ordinates and abscissae respectively, is a

straight line with a slope of 3·6: 1 (actual values range from 2·8–4). A serum, therefore, which gives a 50 p.c. endpoint with virus 1/10^5 will be 1/7 the strength of one giving an endpoint with the standard 1/100 dilution. Since this is the smallest amount of antibody that can be detected by the method a value of 0·15 is the lowest figure given in any of the Tables.

TABLE 2.

Distribution of specific and nonspecific responses to influenza B infection.

	Adults.	Adolescents.	
MIL or GUN titre higher than Lee	6	11	Specific response
LEE higher than MIL or GUN	15	5	Nonspecific response

TABLE 3.

Comparison of haemagglutinin and allantoic titres of a selected group of convalescent sera.

	Corrected allantoic titres.		Haemagglutinin titre.	
Serum.	MIL.	LEE.	MIL.	LEE.
MER	5·1	0·24	250	60
CLA	5·2	0·28	260	70
BAR	0·5	0·85	120	60
KOR	25	< 0·3	640	400
MIL	80	14	800	240
GOD	18	41	480	1,000
ROS	12	22	640	1,280
NIC	8	19	600	1,000
CLE	0·9	< 0·3	120	30
NOL	40	2	1,000	280
Ferret MIL	17	1·8		
„ ELI	27	1·6		

Where the titre of the virus differs from the value $10^{6·3}$ taken as standard, the serum titres are adjusted according to the formula:

$$\text{Log } S^1 = \log S + \frac{\log T - 6·3}{3·6}$$

where S^1 is the corrected serum titre, S the crude serum titre and T the titre of the virus.

In the experiments used to compile Table 3 the virus MIL–B gave titres of $10^{6·2}$ and $10^{5·6}$ at the beginning and end of the series, an average of $10^{5·9}$, calling theoretically at least for correction by dividing the crude titres by $1·29 = \text{antilog } \frac{-0·4}{3·6}$. Virus LEE gave titres of $10^{7·2}$, $10^{6·8}$, $10^{6·4}$ over the period of the experiments, and taking the value $10^{6·8}$ the titres have been multiplied by 1·37. From Knight's (1944) results, the use of 6 embryos per group would not allow results of sufficient accuracy to make small corrections of this sort significant. The figures given are probably accurate to within ± 25 p.c. at 1: 20 level of significance.

Any discussion of the results will, therefore, be confined to major differences. The sera were chosen as representative of various types of antihaemagglutinin activity and, therefore, do not comprise an average cross section. It is probable that the two commonest types of serological response are those exemplified (1) by GOD, ROS, NIC which show high titres against both virus strains, the titre against LEE being approximately twice that against MIL, (2) by MER and CLA from juveniles. These sera show a much higher titre against the homologous MIL virus than against LEE. Antihaemagglutinin and allantoic titres correspond.

The other sera fit more or less into the position of variants of Group 2. KOR in its allantoic reactions is a typically specific juvenile serum from a subject aged 9 but the antihaemagglutinin titre against LEE is unusually high. MIL shows a very high titre against its homologous virus MIL but as well has effective neutralizing power against LEE. BAR is representative of a small group of sera which gave irregular findings in haemagglutination reactions against MIL. In one set of tests 6 sera gave titres against MIL far lower than those obtained in previous or subsequent tests. Two such sera WIL and ALL are included in Table 1. Many other sera tested concurrently showed no such variation. The reason for this variation was not determined. CLE from a young adult shows poor neutralizing activity in the allantoic cavity against both virus strains.

Included in the Table are the findings from allantoic titrations carried out at the same time with sera from ferrets inoculated intranasally with two of the epidemic strains MIL–B and ELI. It will be seen that their activity is closely similar to that of the "juvenile" type sera MER, CLA and NOL.

DISCUSSION.

This work shows the existence of differences in response to influenza B infections of the same general quality as those observed with influenza A. In our first work with the neutralization reaction in the allantoic cavity we found that a proportion of convalescent sera from patients with influenza A showing otherwise normal reactions failed to neutralize when tested in this way. Similar failures are evident in the present series of B infections and the effective "spread" of a serum over related but not serologically identical viruses varies similarly from person to person whether the infection be A or B. In general the sera which are highly effective in neutralizing virus and show a wide "spread" of activity come from adults; with children the reactions tend to be weaker and more specific. The difference is of course far from absolute and we have a considerable number of juvenile type results from adults while in the 14–16 year old boys, providing much of our material, both types occur.

From the practical standpoint the principal question that arises from these results is their bearing on the composition of vaccines for use against influenza B. Published data (Rickard *et al.* 1941, Burnet and Foley, 1940) indicate that there is a fairly high correlation between low antibody level and susceptibility to clinical infection with influenza A. Salk *et al.* (1945), found in a recent study based on the November–December, 1943 epidemic that this correlation was much higher when antibody titres were assessed against a recent strain "Weiss" than when the standard PR8 strain was used. It is reasonable to assume, therefore, that in persons who show a "specific" type of response following infection, an immunizing injection would also be followed by a similarly "narrow" response and protection would be effective only against the subtype of virus used for the vaccine.

If it should become expedient to attempt the preventive immunization of infants against the fatal bronchopneumonias which accompany many influenza epidemics, strict correspondence between the strains used in the vaccine and the epidemic strains might be necessary.

SUMMARY.

Convalescent sera taken after an influenza B infection show specific and non-specific types of response.

In children and adolescents the commonest type is specific, showing a much higher titre both by haemagglutination and allantoic methods against a homologous strain than against the classical strain LEE.

Most adults show equivalent titres against both types of virus but a proportion of specific responses is found.

Influenza B virus strains isolated in 1943 or 1945 form a fairly homogeneous group when tested with specific human sera and differ sharply from LEE.

The significance of these findings in relation to immunization against influenza B is briefly discussed.

REFERENCES.

Andrewes, C. H., Laidlaw, P. P. and Smith, W. (1935): Brit. J. exp. Path., 17, p. 566.
Burnet, F. M. (1943): Austral. J. exp. Biol., 21, p. 231.
Burnet, F. M. and Beveridge, W. I. B. (1943): *Ibid.*, 21, p. 71.
Burnet, F. M., Beveridge, W. I. B. and Bull, D. (1944): *Ibid.*, 22, p. 9.
Burnet, F. M. and Foley, M. (1940): Med. J. Austral., 2, p. 655.
Burnet, F. M. and Lush, D. (1938): Brit. J. exp. Path., 19, p. 17
Burnet, F. M., Stone, J. D. and Anderson, S. G. (1946): Lancet, 1, p. 807.
Friedewald, W. F. (1944): J. exp. Med., 79, p. 633.
Gordon, I. (1942): J. Immunol., 44, p. 231.
Hirst, G. K. (1942): *Ibid.*, 45, p. 285.
Knight, C. A. (1944): J. exp. Med., 79, p. 487.
Rickard, E. R., Horsfall, F. L., Hirst, G. K. and Lennette, E. H. (1941): Pub. Hlth. Rep., 1941, 56, p. 1819.
Rickard, E. R., Thigpen, M. P. and Adams, J. M. (1945): J. infect. Dis., 76, p. 203.
Salk, J. E. (1944): J. Immunol., 49, p. 87.
Salk, J. E., Menke, W. J. and Francis, J., Jnr. (1945): Amer. J. Hyg., 42, p 57.
Stuart-Harris, C. H., Andrewes, C. H., Smith, W., Chalmers, D. K. M., Cowen, E. G. H. and Hughes, D L. (1938): M.R.C. Spec. Rep. Series, No. 228.

THE LIBERATION OF ANEURIN, COCARBOXYLASE, RIBOFLAVIN AND NICOTINIC ACID FROM AUTOLYSING YEAST CELLS

by K. T. H. FARRER

(From the Research Division, Kraft Walker Cheese Co. Pty., Ltd., Melbourne).

(Accepted for publication 29th May, 1946.)

Very little is known concerning the liberation of vitamins from yeast during autolysis. In fact, the only published information seems to be that of Trufanov and Kirsonova (1940) who found that when yeast was allowed to autolyse at 35°–40° C., at pH 4·8 to 5·0, aneurin and riboflavin were set free. A maximum amount of aneurin and riboflavin was obtained after autolysis for 6–10 and 12–24 hours respectively.

The work recorded in this paper describes the rate of liberation of three vitamins of the B complex from the yeast cell during the autolysis of spent brewer's yeast at the optimum temperature. Windisch, Kohlbach and Rothenbach (1928) have shown that this optimum temperature is 50°–55° C., and this has been repeatedly confirmed in these laboratories.

EXPERIMENTAL.

Preparation of Yeast Sample.

A sample of brewer's yeast as received from the brewery was poured through a 50-mesh sieve to remove hop fragments, etc., and washed several times by decantation with cold water. The suspension was then filtered on a Buchner funnel, and a firm yeast cake of about 75 p.c. moisture content was obtained. This was transferred to a screw-capped glass jar and stored in a refrigerator at about 5° C.

Autolysis.

100 gm. yeast cake and 100 ml. of water were mixed together in a 250 ml. Florence flask and incubated in a constant temperature oil bath at 50–52° C. At regular intervals the flask was shaken vigorously and 10 ml. of the suspension were pipetted into a centrifuge tube, mixed thoroughly with 10 ml. of water, and centrifuged. The clear supernatant liquor was transferred to a 25 ml. volumetric flask. The residue was re-dispersed in 6–8 ml. of water, centrifuged, and the clear washings added to the original liquor. The volume was then adjusted to 25 ml.

The pH of the autolysis was the "natural" pH and was between pH 5·5 and 6·0.

Analytical Methods.

Free aneurin. 4 ml. of solution were made up to 50 ml., and 5 ml. aliquots were taken for the thiochrome method. The technique already described (Farrer, 1941) was used in conjunction with the earlier modification due to Wang and Harris (1939) in which the butyl alcohol layer is clarified with ethyl alcohol. The fluorescence was measured with the instrument already described (Farrer, 1941).

Total aneurin and cocarboxylase. 4 ml. of solution were added to about 15 ml. of approx. 0·8 M acetic acid-sodium acetate buffer of pH 4·3, and incubated overnight at 37° C. with the addition of about 100 mg. of takadiastase. The solution was then diluted to 50 ml. and 5 ml. aliquots were assayed for aneurin as already described. As the value obtained in this way represents total aneurin, cocarboxylase was obtained by subtracting the free aneurin figure.

Riboflavin. Riboflavin was determined fluorimetrically using the modification of the Conner and Straub (1941) method employed by Tobin and Rehm (1943) for the examination of fermentation materials and products. Two ml. of the autolysate solution were acidified with 0·5 ml. of glacial acetic acid. One ml. of a freshly prepared 4 p.c. solution of potassium permanganate was then added, and the solution allowed to stand for 3 minutes, after which 1 ml. of 3 p.c. hydrogen peroxide was added and the solution shaken vigorously to expel all bubbles of oxygen and to discharge the colour completely. Ten ml. of re-distilled pyridine were now added and the solution read in the fluorimeter. The riboflavin present was calculated from a graph obtained by treating standard solutions of riboflavin in exactly the same way.

Nicotinic acid. The microbiological assay using *Lactobacillus arabinosus* 17–5 was used. Originally worked out by Snell and Wright (1941) it was later modified by Krehl, Strong and Elvehjem (1943) and Barton-Wright (1944). The details set down by Barton-Wright were followed except that the casein hydrolysate was neutralized with barium hydroxide alone and not partly with litharge. For culture of the inoculum, the organism (obtained from the American Type Culture Collection and carried in a glucose-yeast-agar stab) was grown on the basal medium used for riboflavin assays (Greene and Black, 1943), to which riboflavin had been added. Five ml. of the autolysate solutions were made up to various volumes so that 1 ml. of the resulting solutions contained 0·05 μg. of nicotinic acid, and the assay was carried out in triplicate at the three different levels given by 1, 2 and 3 ml. of the solution. The results have been calculated by the "slope-ratio" method of Wood (1945).

Total solids. Five ml. of solution were pipetted into a tared aluminium dish with a tightly fitting lid, and evaporated to dryness on a water bath. The solids were then dried to constant weight in an air oven at 100–102° C.

RESULTS.

No good purpose would be served by recording all the results obtained. Those recorded in Figs. 1 to 5 are typical of results obtained with yeasts from five different sources.

DISCUSSION.

It is clear that the liberation of vitamins from yeast cells autolysing at the optimum temperature is very rapid. Thus, from the curves presented herein, it is seen that the longest time required for complete liberation was about 80 minutes. It has been found that this time varies from sample to sample, and even in the same sample the time required is shortened by storage, as will be referred to later. However, the longest time required for complete liberation of vitamins from spent brewer's yeast autolysing at 50°–52° C. was between 2¾ and 3 hours. This is very much shorter than that stated to be necessary by Trufanov and Kirsonova.

It can be seen from the graphs that the curves for the liberation of the various vitamins are of the same general form. There is a slow liberation at the beginning of the autolysis gradually leading to a much more rapid rate of liberation, and the relation between autolysis and the amount of vitamin liberated becomes linear. This slow liberation corresponds with the latent period before autolysis begins.

Schryver, Thomas and Paine (1927), studying the keeping qualities of brewer's seed yeast at 20° C., reported a latent period which preceded autolysis. Later, Drews (1936), in studies on the autolysis of various yeasts at 4° and 17° C., encountered a similar phenomenon. Spent brewer's yeast autolyses readily at the optimum temperature of 50°–52° C. but, even so, there is a latent period of up to an hour before autolysis really commences. Fig. 6 is a typical autolysis curve for such yeasts. The length of the latent period varies according to the yeast and its history.

In this work determinations of total solids show clearly the occurrence of a latent period which diminished from about 42 minutes, in the case of fresh yeast, to 35 and 15 minutes respectively as the yeast was stored for three and seven days. Reference to the vitamin liberation curves (Figs. 1, 2, 3 and 5) shows that the beginning of the linear portion corresponds fairly closely with the end of the latent period in each case. In other words, the liberation of the vitamins is closely associated with autolysis; they are not liberated from the cell before autolysis begins, but when autolysis has really started they are liberated quickly and at a steady rate. Confirmation of this was obtained by a similar study of the liberation of aneurin on autolysis from a baker's yeast with a latent period of three hours. It should be pointed out that the nicotinic acid results recorded in Fig. 5 were obtained from a sample of yeast different from that used for the aneurin, riboflavin and total solids studies recorded herein.

The slow autolysis of yeast on storage is well-known (Windisch *et al.*, 1928; Drews, 1936), and the influence of storage on the liberation of the vitamins from

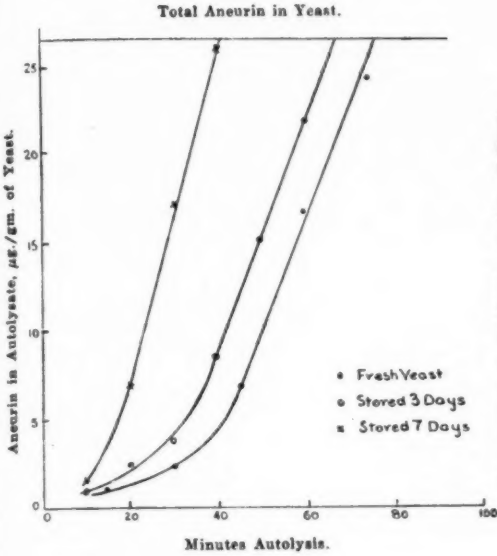


Fig 1.

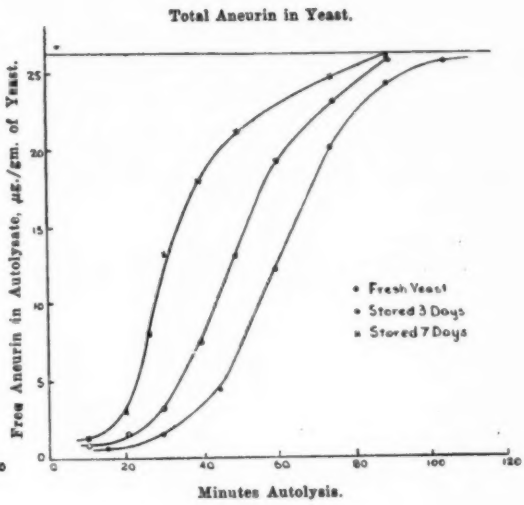


Fig. 2.

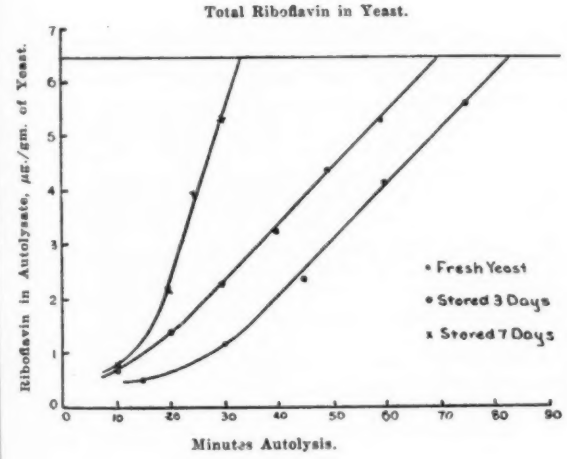


Fig. 3.

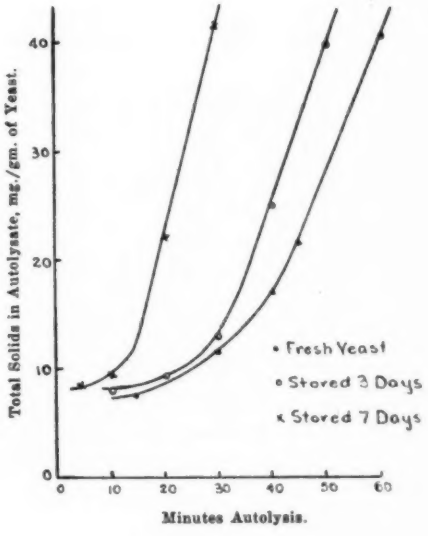


Fig. 4.

the cells, when the stored yeast is autolysed, is clearly seen from the graphs; the longer the storage period, the faster the vitamin liberation on subsequent autolysis.

It is interesting to note, too, that during autolysis the phosphatase of the yeast hydrolyses cocarboxylase (aneurin pyrophosphate) to free aneurin. It can readily be demonstrated that the enzymic hydrolysis of cocarboxylase is a unimolecular reaction, as would, of course, be expected; and if, in the present work, the logarithm of the concentration of cocarboxylase remaining in the autolysate were plotted against time of autolysis, the resulting curve was linear also —but only after the latent period, as measured by the total solids, had elapsed.

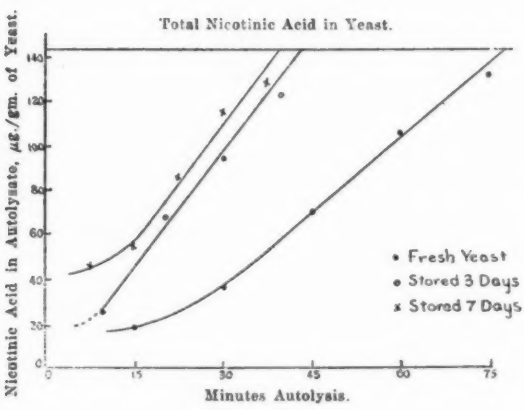

Total Nicotinic Acid in Yeast.
Nicotinic Acid in Autolysate, μg./gm. of Yeast.
• Fresh Yeast
• Stored 3 Days
x Stored 7 Days
Minutes Autolysis.

Fig. 5.

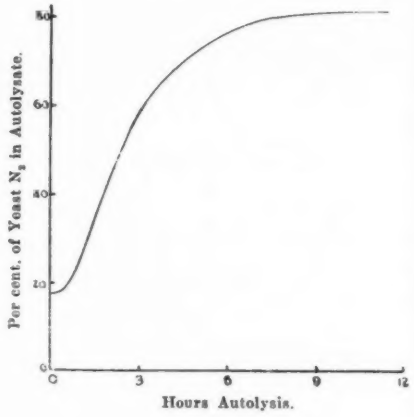


Fig. 6.

It would appear from the curves (Figs. 1 and 2) that the yeast phosphatase, too, can diffuse out of the cell quite rapidly, as the hydrolysis of the cocarboxylase has continued normally after all the vitamin B_1 (i.e. free aneurin plus cocarboxylase) has diffused into the external medium. In the course of this work, however, some evidence for believing that this process is not invariable was obtained. With one sample of yeast, the figures for free and total aneurin were exactly the same in every sample of the autolysate right up to the complete liberation at just under three hours' autolysis. In this case the cocarboxylase was apparently hydrolyzed inside the cell itself. On another occasion, all the vitamin B_1 had been liberated in $1\frac{1}{2}$ hours, but at this point the free aneurin remained constant for about an hour and then very slowly increased until the hydrolysis of the cocarboxylase was complete in about 15 hours. These two samples represent extreme cases. By far the greater number of yeasts examined behaved in the way described in this paper.

It is worth recording that prolongation of autolysis to 24 hours had no effect whatever on the aneurin content of the autolysate.

SUMMARY.

The liberation of total aneurin, riboflavin, and nicotinic acid from yeast autolysing at the optimum temperature of 50°–52° C. is a linear function of time when once autolysis has commenced, and in general was complete in not more than 2½ hours in the yeasts studied.

Slow liberation at the beginning of autolysis is a function of the latent period which precedes autolysis.

The latent period and the time required for complete liberation are both shortened by preliminary cold storage of yeast.

Cocarboxylase is hydrolysed to free aneurin during autolysis, and the latter is unaffected by continuation of the autolysis up to 24 hours.

Acknowledgments. The author thanks the Directors of the Kraft Walker Cheese Company Proprietary Limited for permission to publish this paper, and gladly acknowledges the assistance of Mr. R. G. Miller in the experimental work on aneurin and riboflavin. He is especially indebted to Miss M. Dick for her extensive help in connection with the nicotinic acid assays.

REFERENCES.

Barton-Wright, E. (1944): Biochem. J., 38, p. 314.
Conner, R. T. and Straub, G. J. (1941): Ind. eng. Chem., Anal. Ed. 13, p. 385.
Drews, B. (1936): Biochem. Z., 288, p. 207.
Farrer, K. T. H. (1941): Austral. chem. Inst. J. Proc., 8, p. 22.
Greene, R. D. and Black, A. (1943): J. Amer. pharm. Assoc., 32, p. 217.
Krehl, W. A., Strong, F. M. and Elvehjem, C. A. (1943): Ind. eng. Chem., Anal. Ed. 15, p. 471.
Schryver, S. B., Thomas, E. T. and Paine, S. G. (1927): J. Inst. Brew., 33, p. 120.
Snell, E. E. and Wright, L. D. (1941): J. biol. Chem., 139, p. 675.
Tobin, L. C. and Rehm, J. B. (1943): J. Bact., 45, p. 32.
Trufanov, A. V. and Kirsonova, V. A. (1940): Biokhimiya, 5, pp. 234–9 (Brit. Abst. A., 1941, p. 797).
Wang, V. L. and Harris, L. J. (1939): Biochem. J., 33, p. 1356.
Windisch, W., Kohlbach, P. and Rothenbach, E. F. (1928): Woch. Brau., 45, pp. 251–6, 261–5, 281–5, 298–302.
Wood, E. C. (1945): Nature, 155, p. 632 (see also Analyst, 71, p. 1).

CYSTINE, METHIONINE AND SULPHATE EXCRETION IN CYSTINURIA

by JOAN B. CLELAND

(From the Institute of Medical and Veterinary Science, Adelaide).

(Accepted for publication 8th April, 1946.)

A number of experiments have been carried out on the urinary excretion of cystinurics (Andrews *et al.*, 1938; Brand, Cahill and Harris, 1935; Cleland, 1945; Hess and Sullivan, 1942; Lewis *et al.*, 1936; Lough *et al.*, 1941). In all these cystinurics the excretion of cystine was about half a gram or more each day. Sullivan and Hess (1936) found that the cystine excretion of five normal individuals was about 100 mg. per day; and another normal individual examined by them (Hess and Sullivan, 1942), excreted about 150–200 mg. per day. This paper reports results obtained from a man who excreted about 250–350 mg. per day.

EXPERIMENTAL DETAILS.

History.

The subject of these studies was a man 65 years of age who was admitted to hospital on the 4th September, 1945, with a maculo-papular rash of unknown origin. His temperature was 103° F, and blood cultures and agglutinations were negative. On the 9th September he began to vomit and developed intractable hiccough. Urine examined on the following day was loaded with cystine crystals. While in hospital prior to this the urine was alkaline and fairly concentrated (S.G. 1,030–1,025) but the centrifuged deposit was not examined. On the 11th September he received sulphathiazole with alkalis and copious fluids, and this treatment was continued until the 17th September. The first 24-hour specimen of urine for analysis was collected on the 19th September.

Diet.

The patient's basal diet was such that on each day he ate 2 eggs, 1 serve (3 oz.) of meat, 1 pint of milk and an unlimited amount of cereals, fruit and vegetables. The total protein intake was about 1·2 gm. per kilo body weight.

The amino acids given for experimental purposes were taken in gelatine capsules with breakfast on the morning of the day indicated.

Cystine, methionine or cysteine hydrochloride was given in such amounts that the intake each day corresponded in sulphur content to 2 gm. cystine. The cystine was isolated from sheep's wool and the cysteine hydrochloride was prepared from the cystine by reduction with Sn and HCl. The methionine was made available through the courtesy of Mr. H. R. Marston.

Five gm. glycine (May and Baker) were given on two consecutive days (cf. Hess and Sullivan, 1942) but unfortunately only 3·2 gm. alanine (B.D.H.) were available and this was given in a single dose.

The two eggs in the basal diet were replaced on October 15th and 16th by 14 gm. gelatine and on October 31st and November 1st by 16 gm. casein, each of which contain an equivalent amount of protein. In addition to the basal diet, 14 gm. gelatine were eaten on October 19th and October 20th; 2 additional eggs on October 23rd and October 24th and 16 gm. casein on October 27th and October 28th. As this was not accompanied by a marked rise in urinary nitrogen there must have been a corresponding reduction in the consumption of vegetable proteins.

Methods.

The first specimen of urine collected was alkaline and the specific gravity 1,020. No cystine crystals could be detected, and during the whole series of experiments they were only occasionally present in the centrifuged deposit even though in some cases the pH fell below 6 and the urine

was very concentrated. Efforts made to obtain crystals by acidification, concentration and hydrolysis were not successful.

The daily excretions of nitrogen and of cystine, methionine and sulphate sulphur were determined under various conditions. In addition the content of cystine was determined in 24-hour urines from one daughter and two grand-children; and fasting urine specimens from his two other daughters and two grand-children were examined for the presence of cystine crystals.

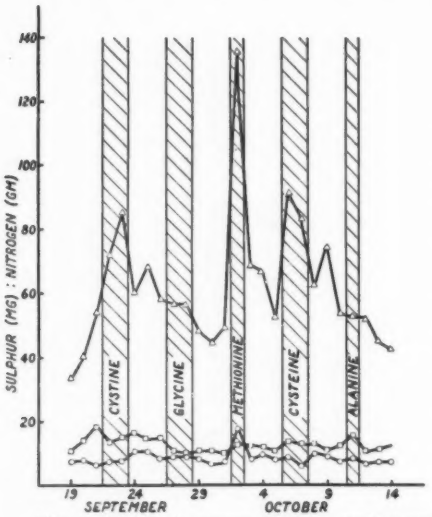


Fig. 1. Ratio of urinary sulphate, cystine and methionine sulphur to total urinary nitrogen excreted each day.

△ Sulphate sulphur (mg.) : Total nitrogen (gm.).

□ Cystine sulphur (mg.) : Total nitrogen (gm.).

○ Methionine sulphur (mg.) : Total nitrogen (gm.).

The urine was collected under toluene and the methods used were those described in a previous paper (Cleland, 1945), except that a photo-electric colorimeter (photelometer-Cenco) was used for the final reading in the Lugg modification of the Sullivan procedure for estimating cystine (Lugg, 1933). Csonka *et al.* (1944), have studied the transmission curve of the red-coloured cystine complex and found that maximum absorption occurred at 505 mμ. At this point transmission through a solution of the unchanged naphthoquinone reagent was almost complete if the concentration was less than 0·3 p.c. In these experiments, using a filter which transmitted light between 450 mμ and 550 mμ and using 0·3 p.c. naphthoquinone reagent in place of the more concentrated 0·02 M solution used by Lugg, the blank solution containing only reagents gave 94 p.c. transmission. Results obtained by using the photelometer could be read more easily and rapidly than with a colorimeter, and gave consistent results.

RESULTS AND DISCUSSION.

Results are shown in Tables 1, 2 and 3 and Fig. 1. The excretions of extra cystine, methionine and sulphate following the ingestion of certain amino acids were calculated for the two days on which they were fed, and for the following day. Control values were obtained from the two preceding the test.

Values used for comparing the effect of different proteins were obtained by finding the average excretion for the two days on which the protein was fed and the following day. Results obtained on October 14th, 18th and 22nd were used to calculate the excretion on the basal diet.

As the total nitrogen excretion varied somewhat about a mean, the results have also been given as the ratio of sulphate, cystine and methionine S respectively per gm. of nitrogen excreted per day (Fig. 1 and Table 3).

Sulphur-containing Amino Acids.

As with other cystinurics the ingestion of cystine itself was not followed by an increase in cystine excretion, but there was a marked rise in urinary sulphate during the following 24 hours which amounted to 36 p.c. of the ingested amino acid.

The response of other cystinurics to methionine has been variable. In some cases there has been an increase in cystinuria (Brand, Cahill and Harris, 1935; Cleland, 1945; Hess and Sullivan, 1942; and Lewis *et al.*, 1936); in one case cystinuria increased only when the protein intake was low (Lough *et al.*, 1941), and in another methionine had no demonstrable effect (Hess and Sullivan, 1942). In the present case there was a slight rise in the ratio of cystine sulphur to total nitrogen excreted, and 2 p.c. of the test dose was recovered as cystine. These results are of the same order as those obtained by Hess and Sullivan (1942) for a normal individual and the difference is therefore insignificant. Unfortunately only 2·5 gm. methionine were available, and it is possible that more clear-cut results would have been obtained if more methionine had been given on the following day. However, there is no doubt that a large proportion of the methionine was oxidized completely, as the extra sulphate obtained would account for 66 p.c. of the ingested dose. Another 12 p.c. of the methionine was recovered as the unchanged amino acid, a value

similar to that obtained by Andrews and Randall (1935) and Cleland (1945). These results would indicate that methionine, except possibly in large doses, does not affect the degree of cystinuria in this subject on a moderate protein intake.

TABLE 1.

Influence of protein and S-containing amino acids on urinary sulphur in cystinuria.

Date.	Substance fed.	Volume. ml.	Total N. gm.	Sulphur (gm.). Cystine.	Methionine.	Sulphate.
Sept. 19		680	7·1	0·08	0·06	0·24
20		1,310	8·3	0·12	0·07	0·33
21		960	3·2	0·06	0·02	0·17
22	2 gm. cystine	880	5·2	0·07	0·04	0·37
23	2 „ „	970	4·5	0·07	0·03	0·38
24		1,300	6·2	0·10	0·07	0·37
25		730	3·9	0·06	0·04	0·27
26		940	4·5	0·07	0·04	0·26
27	5 gm. glycine	910	6·1	0·07	0·06	0·34
28	5 „ „	910	7·9	0·08	0·08	0·44
29		710	4·2	0·05	0·04	0·20
30		1,330	6·5	0·07	0·04	0·30
Oct. 1		1,520	7·2	0·07	0·05	0·37
2	2·5 gm. methionine	1,430	6·4	0·08	0·12	0·66
3		970	5·4	0·07	0·05	0·37
4		680	5·9	0·07	0·06	0·40
5		780	6·8	0·08	0·05	0·36
6	2·7 gm. cysteine hydrochloride	770	5·7	0·08	0·05	0·52
7	2·7 gm. cysteine hydrochloride	1,630	9·3	0·12	0·06	0·78
8		1,010	5·7	0·08	0·06	0·35
9		520*	3·8	0·04	0·04	0·29
10		1,230	6·7	0·08	0·05	0·36
11	3·2 gm. alanine	1,370	6·6	0·11	0·06	0·35
12		1,150	8·0	0·09	0·05	0·43
13		1,210	8·2	0·09	0·06	0·37
14		1,220	7·0	0·09	0·05	0·30
15	14 gm. gelatine replaced 2 eggs in basal diet	1,360	6·9	0·10	0·06	0·33
16	14 gm. gelatine replaced 2 eggs in basal diet	1,480	8·4	0·11	0·06	0·50
17		1,100	6·8	0·09	0·06	0·47
18		850	7·3	0·08	0·06	0·46
19	14 gm. gelatine in addition to basal diet	850	7·8	0·07	0·05	0·41
20	14 gm. gelatine in addition to basal diet	850	8·3	0·09	0·06	0·52
21		1,130	7·6	0·10	0·06	0·41
22		750	6·2	0·06	0·06	0·42
23	2 eggs in addition to basal diet	1,250	6·7	0·09	0·07	0·51
24	2 eggs in addition to basal diet	1,850	8·2	0·11	0·06	0·51
25		1,000	6·8	0·07	0·05	0·50
26		1,360	9·5	0·09	0·05	0·55
27	16 gm. casein in addition to basal diet	1,130	7·5	0·08	0·06	0·45
28	16 gm. casein in addition to basal diet	1,460	8·5	0·11	0·06	0·51
29		1,140	8·2	0·11	0·05	0·68
30		1,650	8·3	0·14	0·04	0·44
31	16 gm. casein replaced 2 eggs in basal diet	1,350	7·4	0·14	0·08	0·65
Nov. 1	16 gm. casein replaced 2 eggs in basal diet	700	5·9	0·11	0·06	0·72
2		1,000	6·7	0·14	0·07	0·76
3		1,080	9·1	0·11	0·06	0·40

* Specimen incomplete.

TABLE 2.

Extra sulphur excreted after various amino acids.

Amino-acid fed.	Amount. (gm.).	Average nitrogen. (gm.).	Extra sulphur excreted as cystine. gm.	P.c.	methionine. gm.	P.c.	sulphate. gm.	P.c.	Amino-acid recovered. P.c.
Cystine	4	5·3	0	—	0	—	0·38	36	36
Glycine	10	6·1	0		0·05		0·19		
Methionine	2·5	5·9	0·01	2	0·07	12	0·35	66	80
Cysteine hydrochloride	5·4	6·9	0·05	5	0	—	0·52	49	54
Alanine	3·2	7·3	0·02		0·01		0·06		

Cystinuria is regarded as being due to a defect in cysteine metabolism, and with one exception (Hess and Sullivan, 1942), cysteine hydrochloride has caused a definite increase in cystine excretion in those cystinurics studied. In this case the rise in cystinuria after taking cysteine hydrochloride amounted to only 5 p.c. of the ingested acid, and as this increase in excretion was accompanied by a corresponding rise in urinary nitrogen, it might be due to an increase in metabolism as a whole rather than to a specific increase in cystine. The amount of extra cystine recovered after feeding with cysteine hydrochloride was so small that it is unlikely that this man has any marked inability to handle it. The fact that the sulphate excretion (49 p.c.) was higher than that obtained after an equivalent amount of cystine (36 p.c.) suggests that oxidation to sulphate is carried out more readily than with cystine itself.

Glycine and Alanine.

Hess and Sullivan (1942) gave glycine and alanine to two cystinurics and one normal subject, and found that in one of the cystinurics there was a marked increase in cystinuria following alanine, and a smaller one after glycine. The other cystinuric and the normal individual were not affected. When 5 gm. glycine were given to the present subject there was a marked rise in urinary sulphate and methionine, but no alteration in cystine excretion. When these values are compared with the concurrent nitrogen excretion, however, it is found that there was an equivalent rise in urinary nitrogen, or, in other words, the increase was due to an increase in protein metabolism as a whole and can probably be attributed to the specific dynamic action of the glycine. There seems no reasonable explanation of why the cystine excretion was not also raised, unless the presence of glycine alters the direction of methionine metabolism or increases the demand for cystine.

When alanine was given there was a definite increase in urinary cystine and sulphate but no alteration in the methionine. The rise in sulphate was accompanied by a proportional rise in nitrogen, but the cystine excretion per unit of nitrogen was higher after alanine than after any of the other amino acids tried, and more extra cystine was obtained after 3·2 gm. alanine than from 2·5 gm. methionine, though not as much as was obtained after a total of 5·4 gm. cysteine hydrochloride given on two consecutive days. This specific effect of alanine is difficult to explain. The recent work of Binkley and du Vigneaud (1942) and Stetten (1942) has shown that methionine is first demethylated to homocysteine which reacts with serine to form cysteine. It is possible that if alanine is the source of serine and if serine were a limiting factor in the rate of this reaction, then alanine might cause an increase in cysteine production. This does not seem very likely. Fromageot *et al.* (1941), working with liver from dogs, came to the conclusion that the following reaction occurred:

$$3 \text{ cysteine} \rightleftharpoons \text{cystine} + H_2S + \text{alanine}.$$

Smythe (1942) was unable to confirm the equation, and his results were more in accord with:

$$\text{cysteine} \rightleftharpoons \text{pyruvic acid} + H_2S + NH_3.$$

However, he (Smythe, 1945) considers that the production of some alanine may also occur. Smythe and Halliday (1942), too, have shown that when H_2S containing radioactive sulphur is included with the cysteine and the enzyme preparation some of the tagged sulphur is found in the cysteine, indicating that the reaction is reversible. If so, the presence of large amounts of alanine might not only limit the removal of cysteine but actually increase its production.

Variations of Protein Intake.

If methionine and cysteine are the source of urinary cystine then it would be expected that an increase in protein intake, or of the proportion of those proteins rich in methionine, would be followed by an increase in cystine excretion. The protein intake was therefore varied by substituting gelatine (poor in cystine and methionine) or casein (poor in cystine) for eggs (rich in both cystine and methionine) in amounts which contained approximately the same quantity of protein. A higher intake of animal protein (and incidentally of cystine and methionine) was

obtained by incorporating an extra 2 eggs in the basal diet. This did not result in any appreciable increase in urinary nitrogen and it can therefore be assumed that the eggs replaced some of the vegetable protein.

TABLE 3.

Effect of different proteins on excretion of S-containing amino acids.

Date.	Added protein.	Average urinary N.	Average daily increase in diet of cystine. (gm.).	Average daily increase in diet of methionine. (gm.).	Average daily excretion of cystine. (gm.).	Average daily excretion of methionine. (gm.).	Average excretion per gm. nitrogen of cystine S. (mg.).	Average excretion per gm. nitrogen of methionine S. (mg.).
Oct. 15–18	14 gm. gelatine	7·4	0·01	0·11	0·38	0·28	13·5	8·0
Oct. 31–Nov. 1	16 gm. casein	6·7	0·06	0·56	0·47	0·33	18·8	10·4
Oct. 14, 18, 22	2 eggs	6·9	0·27	0·59	0·28	0·26	11·2	8·3
Oct.19–21	2 eggs + 14 gm. gelatine	7·9	0·28	0·70	0·31	0·26	10·5	7·0
Oct. 27–29	2 eggs + 16 gm. casein	8·1	0·33	1·15	0·38	0·26	12·6	7·0
Oct. 23–25	4 eggs	7·2	0·54	1·18	0·34	0·28	12·5	8·3

The estimated differences in cystine and methionine content of the diet during the different periods can be seen in Table 3. These were based on the amino-acid content of proteins given by Block and Bolling (1944). An examination of the same Table shows that the lowest values were obtained for both cystine and methionine excretion when the basal diet—2 eggs, one serve of meat and 1 pint of milk, with cereals and fruit and vegetables—was consumed. The addition of gelatine to this was not followed by any appreciable alteration in the amino-acid excretion but, as would be expected, the cystine and methionine excretion per gram of nitrogen was reduced, the rise in urinary nitrogen being attributed to the specific dynamic action of glycine. On the other hand, when the cystine and methionine content of the diet was reduced by substituting gelatine for eggs, the excretion of cystine was greater than and the methionine the same as when 4 eggs (which contain about 1 gm. more methionine and ½ gm. more cystine) were included in the diet, whether the results were determined as extra amino-acid or amino-acid per gm. of nitrogen excreted. Again, when 2 of the 4 eggs were replaced by 16 gm. casein, the methionine excretion was a little lower but the cystine excretion higher, although the methionine intake was unaltered and the cystine intake reduced.

Highest values for methionine and cystine excretion (either as total excretion or per gm. of nitrogen) were obtained when the diet contained 16 gm. casein but no eggs, or, in other words, about 0·6 gm. of methionine but little cystine.

These results show that although cysteine and methionine may be the source of urinary cystine, the amount excreted is by no means proportional to the intake of these amino-acids. Lewis *et al.* (1936), and Lough *et al.* (1941), have already shown that in some cystinurics less cystine was excreted after a given dose of methionine when the protein intake was high than when it was moderate. Cleland (1945) found in a cystinuric who showed a marked response to both methionine and cysteine hydrochloride that alterations in the intake of S-containing amino-acids by varying the type of protein consumed or the total protein intake were not accompanied by equivalent alterations in cystine and methionine excretion.

From these figures it would appear that eggs contain some factor which depresses cystinuria in this individual. Probably its action is on methionine metabolism, for cystine excretion was greatest when in the absence of eggs the diet contained appreciable amounts of methionine. It is possible that cystine itself is responsible for some of this effect, for although ingested cystine did not alter the excretion of cystine, there was a tendency for the ratio of urinary cystine to nitrogen to fall (Fig. 1). It should, however, be remembered that whereas gelatine and casein are almost pure protein, eggs contain appreciable amounts of fat and other substances, some of which (e.g. choline or some of the vitamins) may be involved in methionine metabolism.

It has been suggested, (Cleland, 1945), that a high serine intake may prevent normal cysteine destruction while on a moderate or low protein diet cysteine production from methionine may be limited by the serine content of the diet. A rough estimation of the serine content of gelatine and casein, based on figures obtained by Stokes and Gunness (1945), shows that 14 gm. gelatine contain about 40 mg. serine, and 16 gm. casein about 100 mg. serine. The serine content of eggs has not been determined. If, then, serine were the limiting factor, only about 0·10 gm. cystine could be derived from the 560 mg. of methionine in the casein used. In actual practice the difference in the cystine excretion obtained when gelatine and casein were fed in the absence of eggs amounted to about 0·09

gm. Similarly one would expect that if methionine oxidation to cysteine were blocked by lack of serine more methionine would appear in the urine. Results obtained using casein would fit in with this hypothesis. When, however, eggs were included in the basal diet, the methionine excretion fell although the intake increased. This could be explained by assuming that eggs contain appreciable amounts of serine. On the other hand, there was no concurrent increase in cystine excretion which should have occurred if serine were the only factor involved. It can therefore be assumed that if serine is a factor which influences the cystine excretion in this cystinuric, it is not the only one other than the cystine and methionine content of the diet which is involved.

As the results obtained from this cystinuric differ quite markedly from those found in the majority of cases, they suggest the possibility that the two types may differ in aetiology. There seems no doubt that in most cystinurics there is an inherent inability to utilize cysteine normally, but in this case the evidence does not justify a similar conclusion. It is, of course, quite possible that the man has only a very mild degree of cystinuria and that he is able to utilize cysteine under all but exceptional circumstances, as, for example, when he became seriously ill and dehydrated. Another, and equally plausible explanation is that the condition is due not to a metabolic defect but to an abnormally low renal threshold. In this respect it is interesting to note that of his three relations examined, two—a daughter and grand-daughter (aged 12) excreted relatively large amounts of cystine (160 mg. and 140 mg. per day respectively) though these are still considered to be within the normal range. The other grand-daughter excreted about 60 mg. per day. It was impossible to examine 24-hour specimens of urine from other members of the family, but a rough colorimetric test on single specimens of urine showed that little cystine was present. No crystals of cystine were found in any of the specimens examined.

SUMMARY.

Total nitrogen and cystine, methionine and sulphate sulphur have been determined in the urine of a mild cystinuric who excreted about 250–350 mg. cystine per day.

When cystine, methionine or cysteine hydrochloride was taken by mouth there was a rise in urinary sulphate but no significant increase in cystine excretion. Twelve p.c. of ingested methionine could be recovered unchanged in the urine.

Glycine increased urinary nitrogen, methionine and sulphate in corresponding amounts, but did not affect the cystine excretion.

Alanine caused an equivalent rise in methionine, sulphate and total nitrogen, but the increase in cystine was relatively greater.

Cystine and methionine excretion were least when the basal diet, consisting of two eggs, one serve of meat, one pint of milk, cereals, fruit and vegetables was consumed; and greatest when 16 gm. casein replaced the two eggs in the basal diet. Values intermediate between these two extremes were obtained when the protein supplement to the basal diet (arranged in order of increasing cystine excretion) was 14 gm. gelatine; two additional eggs; 16 gm. casein. When 14 gm. gelatine replaced the two eggs in the basal diet, the results were of the same order as those obtained with the basal diet supplemented with 16 gm. casein.

The urine of two of the three relatives examined contained more than the average content of cystine, though the values still lay within the normal range.

Possible interpretations of these results are discussed.

REFERENCES.

Andrews, J. C., Andrews, K. C. and Runtenber, C. B. (1938) : Proc. Amer. Soc. biol. Chem., J. biol. Chem., 123, iii.
Andrews, J. C. and Randall, A. (1935) : J. clin. Invest., 14, p. 517.
Binkley, F. and du Vingneaud, V. (1942) : J. biol. Chem., 144, p. 507.
Block, R. J. and Bolling, D. (1944) : J. Amer. diet. Assoc., 20, p. 69.
Brand, E., Cahill, G. F. and Harris, M. M. (1935) : J. biol. Chem., 109, p. 69.
Cleland, J. B. (1945) : Austral. J. exp. Biol., 23, p. 273.
Csonka, F. A., Lichenstein, H. and Denton, C. A. (1944) : J. biol. Chem., 156, p. 571.
Fromageot, C., Wookey, E. and Chaix, P. (1941) : Enzymologia, 9, p. 198. As quoted by Smythe (1945).
Hess, W. C. and Sullivan, M. X. (1942) : J. biol. Chem., 142, p. 3.
Lewis, H. B., Brown, B. H. and White, F. R. (1936) : *Ibid.*, 114, p. 171.
Lough, S. A., Perilstein, W. L., Heinen, H. J. and Carter, L. W. (1941) : *Ibid.*, 139, p. 487.
Lugg, J. W. H. (1933) : Biochem. J., 27, p. 668.
Smythe, C. V. (1942) : J. biol. Chem., 142, p. 387.
Smythe, C. V. (1945) : Advances in Enzymology, 5, p. 237.
Smythe, C. V. and Halliday, D. (1942) : J. biol. Chem., 144, p. 237.
Stetten, De W. (1942) : *Ibid.*, 144, p. 501.
Stokes, J. L. and Gunness, M. (1945) : *Ibid.*, 157, p. 651.
Sullivan, M. X. and Hess, W. C. (1936) : *Ibid.*, 116, p. 221.

THE EFFECT OF SALICYLATES AND THIAMINE DEFICIENCY ON THE THIAMINE CONTENT OF RAT TISSUE

by JOAN B. CLELAND

(From the Institute of Medical and Veterinary Science, Adelaide).

(Accepted for publication 18th April, 1946.)

In a previous experiment (Cleland, 1943), it was found that in the few cases studied, the administration of salicylates was associated with an increase in the amount of thiamine excreted in the urine of humans. Under similar conditions an increase in the excretion of ascorbic acid was noted by Daniels and Everson (1936) and Keith and Hickman (1938), while Samuels *et al.* (1940), found that in rats the increased excretion of vitamin C was associated with a loss of it from the liver and brain. The present paper reports the effect of the administration of salicylates on the thiamine stores of rats.

METHOD.

Experiments were made using both young and adult rats which were fed on either the normal stock diet or a thiamine deficient diet of the following composition:

20	parts	casein
70	„	sucrose
5	„	salt mixture (Harris, 1938)
10	„	dried autoclaved yeast (Evans *et al.*, 1929)
3	„	cod liver oil substitute

Adult rats of the same sex were divided into pairs of approximately the same age and weight. One rat of each pair received by stomach tube 0·5 c.c. 10 p.c. sodium salicylate each day for eighteen days, while the other received 0·5 c.c. distilled water at the same time and under the same conditions The animals were killed after eighteen days and the thiamine content of various tissues was determined.

When young rats (2 months old) were used, the amount of salicylate given had to be reduced to 0·3 c.c. 5 p.c. sodium salicylate given four times a week. This was administered by means of an adapted syringe needle made to the directions given by Ferril (1943). These young rats were fed on either the deficient or the normal stock diet and, as with the adult rats, those on each type of diet were divided into pairs of approximately the same age and weight, one of which received sodium salicylate, the other an equivalent amount of water. Those on the normal diet were killed after receiving treatment for six weeks, while those on the deficient diet were left until they died (4–8 weeks), and the tissues were then removed for analysis.

Brain, liver and kidney were the tissues chosen for analysis because the thiamine content of the two latter has been shown to vary rapidly with alterations in thiamine intake (Ferrebee *et al.*, 1942), and would therefore be more likely to reflect variations in the thiamine status of the rat, while Samuels *et al.* (1940), found that when rats received salicylates there was a loss of ascorbic acid from the liver and the brain.

The fresh tissues were prepared for analysis by the method described by Ferrebee *et al.* (1942), for human tissue, except that smaller amounts of tissue and reagents were used and the tissue was finely ground with acid washed sand instead of using the Waring blendor. Alexander (1943), however, has found that grinding the tissues extracted as much thiamine as did dispersion in the blendor. The determination of thiamine in the final extract was done by the thiochrome method of Wang and Harris (1939).

RESULTS.

The average and range of values obtained for liver, kidney and body weight and for thiamine concentration in liver, kidney and brain for each different group of rats are shown in Table 1. These have been examined statistically.

TABLE 1.

Weight and thiamine content of rat tissues.

No. of rats.	Age.	Received.	Diet.	Weight (gm.). Total.	Liver.	Kidney.	Thiamine concentration (γ/gm.). Liver.	Kidney.	Brain.	
12	adult	water	normal	203	9·04	1·55	3·00	1·78	0·91	Average
				154–276	6·81–11·65	1·40–2·0	1·96–4·83	0·81–3·14	0·58–1·41	Range
12	adult	salicylates	normal	198	9·54	1·56	3·91	2·06	0·97	Average
				148–262	7·76–13·04	1·42–2·24	2·40–7·71	1·26–3·18	0·45–1·48	Range
12	adult	water	thiamine deficient	195	9·35	1·86	0·32	0·39	0·56	Average
				156–255	7·17–13·10	1·57–2·17	0·20–0·43	0·22–0·49	0·39–0·77	Range
12	adult	salicylates	thiamine deficient	198	11·63	2·07	0·40	0·39	0·53	Average
				156–255	8·18–14·79	1·75–2·54	0·29–0·81	0·22–0·73	0·40–0·70	Range
12	young	water	normal	139	8·09	1·12	2·16	2·20	0·83	Average
				124–180	6·15–11·49	0·89–1·48	1·33–3·85	0·69–3·87	0·42–1·18	Range
12	young	salicylates	normal	144	8·47	1·25	2·18	1·94	1·03	Average
				116–171	5·89–11·44	0·92–1·52	1·69–2·79	0·84–2·96	0·45–1·59	Range
12	young	water	thiamine deficient	56	2·49	0·93	0·21	0·17	0·21	Average
				38–80	1·72– 3·23	0·63–1·44	0·12–0·40	0·09–0·33	0·10–0·35	Range
12	young	salicylates	thiamine deficient	63	2·74	1·07	0·15	0·21	0·21	Average
				41–80	2·01– 3·81	0·72–1·27	0·06–0·24	0·11–0·32	0·14–0·36	Range

With adult rats on a normal diet it can be seen that there was little difference between rats receiving salicylates and those receiving water in body weight, kidney weight or thiamine concentration in the brain. The rats receiving salicylates, however, had a significantly greater (P less than 0·01) liver weight (the average increase being about 0·5 gm.) and thiamine concentration in the liver (average increase about 0·9 γ/gm.). The apparently increased concentration of thiamine in the kidney of 0·3 γ/gm. was not statistically significant.

Similarly, in the adult deficient rats there was an even greater increase in liver weight (average 2 gm.) and a smaller, but still significant rise in thiamine concentration in the liver of those animals receiving salicylates, while the other factors studied—body and kidney weight, and thiamine concentration in kidney and brain—were unaltered.

In young rats, however, on both normal and deficient diets, animals receiving salicylates did not differ significantly in any of these particulars, though they tended to be a little heavier. This was accompanied by an equivalent increase in organ weight so that the ratio of organ to body weight remained the same.

In the young rats the rate of development of thiamine deficiency was not affected by the administration of salicylates. It was also found that on the normal stock diet the older rats had a significantly greater (P less than 0·01) concentration of thiamine in the liver than the young rats, the average value obtained from all the adult rats being about 3·5 γ/gm. and from all the young rats about 2·2 γ/gm. In both age groups the concentration was about the same in the kidney (ca. 2·0 γ/gm.) and the brain (ca. 0·9 γ/gm.).

On the other hand, when animals were fed on the thiamine deficient diet the thiamine concentration in the brain of adult rats (ca. 0·5 γ/gm.) exceeded the concentration in other tissues and was significantly greater than the concentration in the brain of the young deficient rats. In the adult rats the concentration in both liver and kidney was about 0·4 γ/gm. while in the young deficient rats the concentration in all three tissues was about 0·2 γ/gm.

As would be expected, in the young deficient rats there was a greater ratio of both liver and kidney weight per gm. body weight than in the control group on the normal diet.

DISCUSSION.

The most significant finding of these experiments is the relatively greater weight of the liver of those adult animals which received salicylates. Macroscopically the livers of these animals were not engorged and sections of three of them showed no histological abnormality and did not differ materially from those of the corresponding control animals. Because the thiamine concentration in these

organs was increased it seems most likely that the greater weight was due to hypertrophy rather than to deposits of fat, water or glycogen which, presumably, would not be associated with the retention of thiamine. It is curious, though, that no records of the effect of salicylates on organ weight can be found in the literature. Samuels *et al.* (1940), in similar experiments on ascorbic acid excretion in the rat, record the organ weight (but not body weight) only of animals which had received salicylates for 1–3 days. It is unlikely that this would be sufficient to provoke hypertrophy, and the results given by them show no significant difference in this respect between treated and untreated animals.

In humans, Kapp and Coburn (1942) found that 80 p.c. of ingested salicylate could be recovered in the urine with the salicyl radicle intact. Of this 55 p.c. was conjugated with glycine and 25 p.c. with glucuronic acid. Assuming that in the rat the liver is the chief site of detoxication, the relative increase in the weight of liver could be attributed to the increased strain thrown on it by the need for salicylate detoxication. Lipschitz and Bueding (1939), have shown that rat liver slices are capable of producing conjugated glucuronates and that production was greatest when pyruvic acid or dihydroxyacetone was used as substrate. As thiamine is necessary for normal utilization of pyruvic acid it is probably required for glucuronate synthesis. Martin *et al.* (1943), found that in rats thiamine deficiency reduced glucuronic acid excretion. Lutwak-Mann (1942), however, failed to find an increased excretion of glucuronates when salicylates were injected into rats, nor did she find that glucuronates were produced *in vitro* by rat liver slices in the presence of salicylate though they were formed when p–hydroxybenzoic acid was used. On the other hand she found that *in vitro* M/10 salicylate affected the carboxylase system and the dismutation between hexosediphosphate and pyruvate. The fact that injections of salicylate were associated with a sudden rise in liver glycogen levels indicates that it is somehow connected with carbohydrate metabolism (Lutwak-Mann, 1942).

The increased concentration of thiamine in rat liver does not suggest an explanation for the increased thiamine excretion found when salicylates were given to several normal people. In fact, the administration of salicylates appeared to increase the efficiency with which thiamine was stored (to a limited extent) in adult rats. This, however, did not occur in young rats, and symptoms of thiamine deficiency occurred just as readily in young deficient rats receiving salicylates as in the control animals which were given water.

The thiamine concentration of the tissues of young deficient rats probably indicates the minimum concentration compatible with life, since these figures were obtained from animals which died of thiamine deficiency. It is of interest to note that under these conditions the concentration in the three tissues studied was about the same—that is, about $0{\cdot}2\gamma$ per gm. In the adult rats in which the deficiency was not nearly so severe, the thiamine concentration in the liver and kidney was not significantly greater than in the young deficient rats, but the concentration in the brain was much higher. This suggests that the brain has first preference on available thiamine and that the content of the brain is less labile than that of other tissues. With a normal diet the concentration of thiamine in the liver was greater than in the kidney which was greater than in the brain, but when the diet was not adequate the greatest loss occurred from the liver and the least from the brain. These results are similar to those obtained by other workers (Ferrebee *et al.*, 1942; Schultz *et al.*, 1939; Sure and Ford, 1942; Sarett and Perlzweig, 1943; Schweigert *et al.*, 1943).

SUMMARY.

Administration of salicylates increased the liver weight per gm. body weight and the thiamine concentration in the liver of adult (but not young) rats, on both normal and thiamine deficient diets. Thiamine concentration in kidney and brain, kidney weight, and the rate of development of symptoms of thiamine deficiency in young rats were not altered by giving salicylates.

In young thiamine-deficient rats the thiamine concentration in brain was lower than in adult deficient rats but the concentration in kidney and liver was

much the same; on a normal diet the concentrations in kidney and brain were similar in adult and young rats, but the thiamine concentration in the liver was significantly higher in adult rats.

Possible interpretations of these results are discussed.

REFERENCES.

Alexander, B. (1943): J. biol. Chem., 151, p. 455.
Cleland, J. B. (1943): Austral. J. exp. Biol., 23, p. 153.
Daniels, A. L. and Everson, G. J. (1936): Proc. Soc. exp. Biol. and Med., 35, p. 20.
Evans, H. M. and Lepkovsky, S. (1929): J. biol. Chem., 83, p. 269.
Ferrebee, J. W., Weissman, N., Parker, D. and Owen, P. S. (1942): J. clin. Invest, 21, p. 401.
Ferril, H. W. (1943): J. lab. and clin. Med., 28, p. 1624.
Harris, L. (1938): "Vitamins and Vitamin Deficiencies", Vol. I; Churchill, London, p. 115.
Kapp, E. M. and Coburn, A. F. (1942): J. biol. Chem., 145, p. 549.
Keith, J. D. and Hickman, E. M. (1938): Arch. Dis. Childhood, 13, p, 125.
Lipschitz, W. L. and Bueding, E. (1939): J. biol. Chem., 129, p. 333.
Lutwak-Mann, C. (1942): Biochem. J., 36, p. 706.
Martin, E. J. and Stenzel, W. (1943): Arch. Biochem. 3, p. 325.
Samuels, L. T., Ritz, N. D. and Poyet, E. B. (1940): J. Pharmac. and exp. Therapy, 68, p. 465.
Sarett, H. P. and Perlzweig, W. A. (1943): J. Nutrition, 25, p. 173.
Schultz, A. S., Light, R. F., Cracas, L. J. and Atkin, L. (1939): *Ibid.*, 17, p. 143.
Schweigert, B. S., McIntire, J. M. and Elvehjen, C. A. (1943): Arch. Biochem., 3, p. 113.
Sure, B. and Ford, Z. W. (1942): J. biol. Chem., 146, p. 241.
Wang, Y. L. and Harris, L. J. (1939): Biochem. J., 33, p. 1356.

CHANGES IN THE CONCENTRATIONS OF CAROTENE AND OF VITAMIN A IN THE BLOOD OF SHEEP GRAZING ON NATURAL PASTURES IN SOUTH AUSTRALIA

by A. W. PEIRCE

(From the Commonwealth Council for Scientific and Industrial Research, Division of Biochemistry and General Nutrition, University of Adelaide).

(Accepted for publication 22nd May, 1946.)

From the evidence at present available it appears that vitamin A alone of all the known accessory food factors is likely to be of importance to grazing sheep in Australia. The sheep derives its vitamin A from the carotenes contained in its fodder. The intake of these precursors of vitamin A greatly exceeds the grazing animal's requirements when the pastures are green but only a small proportion is retained; part of this is used to satisfy the metabolic needs of the animal, the remainder is stored, principally in the liver.

As the forage plants mature, their concentration of carotene decreases. Only a trace remains by the time they are dry, Myburgh (1935 and 1940), Moon (1939), Snyder and Moore (1940), Kemmerer, Fudge and Fraps (1942) and Underwood and Conochie (1943). When sheep forage on dry pastures, as they do in Australia for several months each year, they draw heavily on their reserves. In time of drought, when the period of dry feeding is sometimes extensive, these reserves may become seriously depleted. The length of the period which elapses before symptoms of vitamin A deficiency appear in sheep under conditions of dry feeding will depend on the extent of the initial reserves.

While a prolonged deficiency of vitamin A eventually leads to the death of the animal, whatever the species, this is likely to occur among grazing sheep in Australia only after an extended period of drought. Under such conditions, however, many sheep die from sheer inanition and so the terminal symptoms of vitamin A deficiency are rarely encountered. Indeed, symptoms of an uncomplicated deficiency of vitamin A may not be seen under natural conditions, for they are likely to supervene only at a time when much of the nutritive value of the pasture has been lost. When the pasture is old and dry the concentrations of vitamin A, protein, mineral matter, etc., are all low.

The investigations concerning the relationship of vitamin A to grazing sheep appear to be limited to those carried out in Western Australia by Underwood and Conochie (1941 and 1943) and by Underwood and Curnow (1944). These workers reported the progressive decrease in the reserves of vitamin A in the sheep's liver during the dry period and the very low concentrations of carotene in the dry pasture species and in most of the supplementary feeds which are used when the pastures are dry.

In a previous paper the author, Peirce (1945), reported the effect of intake of carotene on the general health and on concentrations of carotene and of vitamin A in the blood and liver of sheep maintained in pens. The present paper reports the findings from an investigation carried out on grazing sheep in South Australia.

THE EXPERIMENT.

The changes occurring in the concentrations of carotene and of vitamin A in the blood of Merino sheep grazing on the natural pasture of the southern portion and on that of the

pastoral country of the north-west of South Australia were investigated. In the course of these observations some data were obtained which indicated the reserves of vitamin A in the livers of these sheep; these implied the order of the vitamin A reserves in the sheep grazed under similar conditions elsewhere. The main observations were made on sheep which grazed at the Waite Agricultural Research Institute, which is typical of the southern portion of the State. The subsidiary series of observations was carried out on sheep which constituted the experimental animals of a grazing trial which was being conducted by the Waite Institute on Yudnapinna Station, situated 50 miles north-west of Port Augusta.

Investigations on Sheep grazed at the Waite Institute.

In this investigation, eight ewes aged approximately three and a half years at the beginning of the experiment were studied. They were part of the Division's breeding flock; they grazed throughout the year on natural pasture and were provided during the late summer and autumn with a supplementary ration of barley, which contained less than 1γ carotene per gm.

The seasonal conditions in this locality are typical of the Mediterranean climate of southern Australia. Almost 75 p.c. of the mean annual rainfall, 24 inches, falls between May and October. The occasional falls between November and the break of the season in the following April or May are of little or no benefit to the pasture.

On the area where the experimental animals were grazed the pasture consists largely of annual species. These germinate with the first autumn rains, which begin, according to season, between March and May. If the initial falls are early, good growth is made prior to the onset of winter when the production of the pasture is reduced. In the spring a flush of growth occurs. The grasses usually flower in September and by the end of November have shed their seed and dried off. They remain dry until the cycle begins again with the advent of the autumn rains. The sheep thus subsist generally for six months on green grass and for the rest of each year on dry pasture.

The concentrations of carotene and of vitamin A in the plasma of the sheep were determined at intervals of approximately one month. In the twenty-ninth month of the experiment, and thereafter at approximately monthly intervals, one of the sheep was killed, and the concentrations of carotene and of vitamin A in its liver were estimated; all but one of the animals were slaughtered for this purpose.

As it is not practicable to assess that portion of the pasture which the grazing animal selects, the variation of carotene concentration in the pastures was not determined. However, in November, 1942, when the experiment had been in progress for a year, one sample from each of ten species of clover and nine species of grass was collected from adjacent areas; most of the plants were either flowering or about to flower. The green leaves only were analysed.

Investigations at Yudnapinna Station.

The design of this grazing experiment has been described by Woodroffe (1941) and the principal soil and vegetation relationships of the area have been described by Crocker and Skewes (1941). The mean annual rainfall of the area is 7·5 inches but it is extremely variable. It is sporadic and ranges from 18·08 inches to 2·36 inches. The vegetation of the experimental area consists of an open association with myall (*Acacia Sowdenii*) the dominant tree and blue bush (*Kochia sedifolia*) the principal shrub. Various grasses and herbage become prominent temporarily, after the rare effective rains.

As the Station was 250 miles from the laboratory it was not practicable to carry out as frequent or as precise determinations as might be deemed desirable; on several occasions it was not possible to secure samples from all of the sheep. Nevertheless, the results obtained provide a reasonable measure of the status of sheep grazing on pastoral areas of this type.

Blood was collected from twenty-four wethers which were three years of age when the observations began. Samples were taken at intervals of approximately three months over a period of twenty months. On each occasion, the sheep were mustered into a bare yard in the evening and were bled next morning. After somewhat incomplete separation with the aid of a hand centrifuge, the serum, 10 ml., was frozen and stored on solid CO_2. It remained in this condition until analysed, usually two or three days later. Some of the samples at the fifth collection and all at the sixth collection were preserved by the addition of 5 mg. diphenylamine, preliminary tests having shown that the addition of diphenylamine to serum prevents the loss of vitamin A for at least two weeks providing the sample is stored in the dark at temperatures between approximately 20° and 25° C. Diphenylamine had been employed by other workers for preserving the carotene in plant materials. The evidence obtained in these preliminary tests indicated that the vitamin A in plasma is much more stable than is generally believed. No measurable loss of vitamin A was observed in samples of plasma stored in the dark at the above temperature, without added preservative, for one week; loss could be detected, however, in samples stored under these conditions for two weeks.

Samples of liver were collected from seven ration sheep which were killed at intervals during the experiment. Each sample was placed in a weighed amount of 10 p.c. KOH within one hour

of the death of the animal. The sample was then despatched to the laboratory where, on its arrival two or three days later, it was placed in the refrigerator. The concentrations of carotene and of vitamin A were estimated during the ensuing week. Preliminary tests indicated that under such circumstances carotene and vitamin A are not destroyed.

Estimations were also made of the carotene content of a number of typical fodder plants which were collected at each period after the first. These plants were representative of those on which sheep normally graze in this area. They included blue bush, salt bush, bindyi and buckbush, together with herbage, when this was available. Samples of those edible shrubs and trees to which the sheep occasionally had access were also analysed. Eleven different species were sampled regularly and a further five species on one or two occasions only. The leaves and young twigs were stripped off by hand and transferred to weighed amounts of ethyl alcohol. The material thus approximated that eaten by the sheep. The samples were stored in the dark during transport next day to the laboratory. They were then placed in the refrigerator, and remained there until within a week they were analysed for carotene. Little if any loss occurs under such conditions.

Chemical Methods.

The method used for the estimation of carotene and of vitamin A in the blood was that described by Peirce (1945); the corresponding estimations on liver were made by the method of Underwood and Conochie (1941). The phase-separation method of Peterson (1941) was employed for the estimation of the carotene in the pasture plants collected at the Waite Institute but the chromatographic method of Wall and Kelley (1943) was employed for the estimation of the carotene in the forage plants at Yudnapinna; these latter figures are thus not strictly comparable with the former.

RESULTS.

Observations at the Waite Institute.

The concentrations of vitamin A in the blood of individual sheep depastured at the Waite Institute are given in Table 1, and the mean concentrations of carotene and of vitamin A are shown in Fig. 1 in relation to the rainfall and the state of the pastures at various periods during the experiment.

TABLE 1.

Concentration of vitamin A in the blood of grazing sheep at the Waite Institute.

(γ vitamin A per 100 ml. plasma).

Date.	W601	W607	W610	W613	W621	W629	W668	W676	Mean
5/11/41	34	—	37	34	31	42	28	30	34
10/12/41	28	27	31	32	28	33	27	24	29
5/1/42	34	36	—	34	33	38	29	31	34
12/2/42	30	29	28	29	30	29	23	27	28
10/3/42	28	30	32	28	35	32	24	25	29
6/4/42	34	—	36	34	36	40	31	29	34
5/5/42	31	32	32	24	32	34	28	28	30
1/6/42	29	35	32	27	32	40	39	29	33
1/7/42	34	32	29	35	29	31	29	26	31
3/8/42	40	25	31	29	27	29	26	23	29
27/8/42	35	28	28	30	25	37	26	26	29
21/9/42	32	36	45	34	33	44	30	29	35
6/11/42	29	27	30	34	27	31	25	26	29
10/12/42	29	31	34	34	30	33	28	28	31
29/1/43	32	36	44	39	43	39	35	29	37
24/2/43	30	32	37	31	35	37	31	27	33
1/4/43	32	34	34	36	32	37	32	27	33
12/5/43	33	32	29	29	30	43	27	26	31
8/6/43	41	31	—	34	33	39	31	29	34
4/8/43	31	27	42	27	29	28	26	24	29
15/9/43	33	28	48	36	39	36	34	29	35
12/10/43	46	37	42	27	38	49	36	32	38
5/11/43	36	27	36	36	33	39	31	24	33
13/12/43	32	30	32	30	29	31	29	25	30
26/1/44	34	35	39	37	29	40	34	40	36
25/2/44	37	40	34	34	44	38	31	31	36
31/3/44	34	33	28	33	42	38	34	30	34
11/5/44	34	35	30	—	36	—	30	27	32
9/6/44	—	33	35	—	39	—	37	29	35

The results of statistical analysis of the findings indicated that there were no significant differences between the mean concentrations of vitamin A in the blood during consecutive summer and winter periods despite variations of the order of two thousand-fold in the intakes of carotene. There was, however, a slight but significant increase in concentration of vitamin A in the blood throughout the course of the experiment. As the intake of carotene varied considerably from the very low levels in the summer and autumn to high levels in the winter and spring, the steady increase in the concentration of vitamin A in the blood throughout the term of observation would appear to be due to one of two causes. Either the reserves of vitamin A in the liver increase with age and the extent of this reserve influences the concentration in the blood, or some change in the metabolism of vitamin A supervenes as the animal becomes older. The reason is as yet obscure.

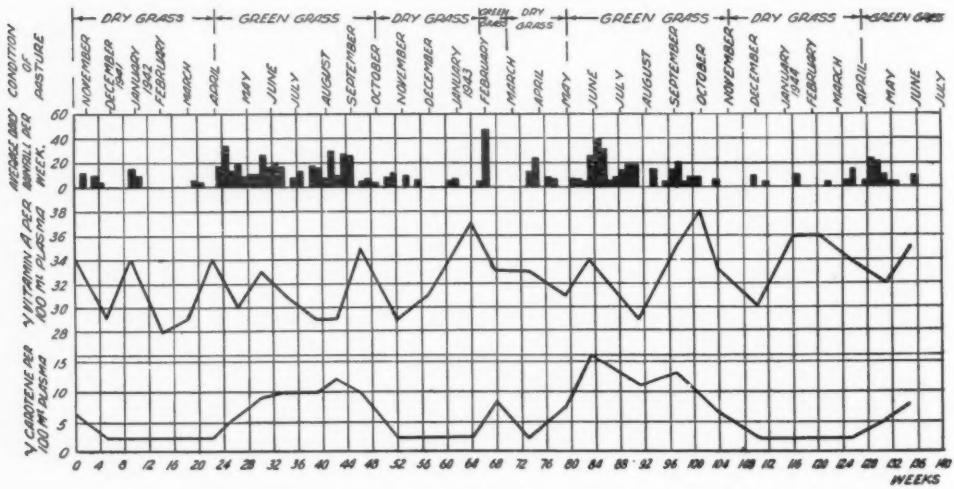


Fig. 1. Mean concentrations of vitamin A and of carotene in the blood of sheep, condition of pasture and mean daily rainfall throughout the experiment.

The carotene in the blood, although low at all times, did tend, however, to be influenced by the intake of carotene; its concentration fell to between 2γ and 4γ per 100 ml. plasma in the periods of dry feeding. In Fig. 1 these "traces" of carotene have been represented as being equivalent to a concentration of 2γ per 100 ml. plasma as the amounts found were close to the limit of sensitivity of the analytical method. The concentration rose to measurable amounts when green grass became available; values ranging between 5γ and 16γ per 100 ml. plasma were observed during this period. Even when at its maximum concentration, however, the carotene represented only about 20 p.c. of the total vitamin A potency of the blood.

TABLE 2.

Concentration of vitamin A in the livers of sheep grazing at the Waite Institute.

Sheep.	Date killed.	Weight of liver gm.	Vitamin A γ/gm. liver (wet wt.).	Vitamin A γ per liver.
W613	29/3/44	640	807	516,000
W629	11/4/44	604	499	301,000
W601	11/5/44	618	322	199,000
W676	8/6/44	1,050	515	541,000
W621	6/7/44	770	327	252,000
W610	10/8/44	808	662	535,000
W607	28/9/44	825	234	193,000

The concentration of carotene in the blood responded rapidly to changes in the intake. The observations towards the end of February and at the beginning of April, 1943, render this clear. Following heavy rains which resulted in a growth of green grass the carotene concentration in the blood rose abruptly from 2γ to 8γ per 100 ml. plasma. By the time the next determination was made, five weeks later, the grass had dried off and the concentration of carotene in the blood had again fallen to below 4γ per 100 ml. plasma.

The concentrations of vitamin A in the livers of the sheep killed at intervals from the twenty-ninth month of the experiment are shown in Table 2.

The concentrations of vitamin A in the livers of the individual sheep varied greatly when considered either in terms of concentration per unit weight or in terms of total amount per liver. The number of sheep examined was small, but nevertheless there is little doubt that the reserves of vitamin A in the livers were always high. Every sheep at the time when it was slaughtered had ample reserves; this was true even for the two sheep killed at the end of summer, in March and April, 1944, when the reserves might have been expected to be at a minimum.

The amount of carotene in the liver was always low. It was found to vary between 2·0γ and 4·0γ per gm. liver (wet weight). This would account for less than 0·5 p.c. of the total vitamin A potency of the liver. It was, in consequence, neglected in the calculation. The concentration of carotene was no lower in the livers of the two sheep killed at the end of summer than it was in those of sheep killed when their intake of carotene was much higher than their total requirements. In the sheep the conversion to vitamin A of the carotene which is retained is thus rapid and efficient.

The concentrations of carotene in the grasses and clovers collected in November, 1942, from areas near the paddock in which the sheep were pastured are set out in Table 3.

TABLE 3.

Concentration of carotene in the green leaves of various species of clover and grass.

Species.	Carotene mg./kg. fresh material.
CLOVERS:	
Red clover, *Trifolium pratense*	700
White clover, *Trifolium repens*	660
Barrel medic, *Medicago tribuloides*	630
Subterranean clover, *Trifolium subterraneum*	600
Burrless burr clover, *Medicago hispida confinis*	600
Snail medic, *Medicago scutellata*	580
Alsike clover, *Trifolium hybridum*	570
Drooping flowered clover, *Trifolium cernuum*	560
Burr medic, *Medicago denticulata*	510
Trifolium lappaceum	410
GRASSES:	
Kentucky blue grass, *Poa pratense*	580
Rhodes grass, *Chloris gazana*	530
Perennial veldt grass, *Erharta calycina*	50[0]
Timothy, *Phleum pratense*	480
Yorkshire fog, *Haleus lanatus*	450
Toowoomba canary grass, *Phalaris tuberosa*	390
Akaroa cocksfoot, *Dactylis glomerata*	370
Hawke's Bay rye grass, *Lolium perenne*	370
Italian rye grass, *Lolium multiflorum*	350

The concentration of carotene was high in the green leaves of the clovers and grasses. The former contained about 30 p.c. more carotene than the latter.

A sheep which consumes 5 kg. of green grass (approximately 1 kg. dry weight) each day would thus take approximately 2 gm. carotene in its fodder when on pastures of this sort. Assuming a value of 1 mg. carotene per kg. which is an average for the concentration in dry grass late in the summer, and a consumption of 1 kg. daily, the intake at this period would be reduced to about 1 mg. carotene or 20γ per kg. body weight daily. Notwithstanding this difference, about two thousand-fold, in intake, no statistically significant difference between the mean concentrations of vitamin A in the blood during the consecutive summer and winter periods was observed (see p. 234).

Observations at Yudnapinna Station.

The results of the determinations of vitamin A in the blood of the sheep grazed throughout the experiment at Yudnapinna Station are set out in Table 4.

TABLE 4.

Concentration of vitamin A in the blood of sheep grazing at Yudnapinna.

(γ vitamin A per 100 ml. serum).

Sheep.	20/4/43.	28/10/43.	8/2/44.	2/5/44.	1/8/44.	19/12/44.
114	35	31	27	30	32	—
134	25	32	22	25	15	14
137	—	30	24	30	25	15
141	27	39	31	33	38	—
158	27	32	27	25	29	19
161	—	32	26	30	31	18
162	31	41	32	34	37	22
163	30	40	30	44	32	25
167	24	28	24	30	—	20
170	29	36	27	32	35	19
172	26	41	30	37	31	24
173	30	34	24	34	—	21
174	24	28	27	26	—	17
176	32	33	25	39	—	—
177	26	32	25	31	30	18
178	25	30	23	23	23	17
179	24	30	25	29	—	18
183	26	27	21	26	—	18
184	32	37	25	31	—	19
187	28	—	25	27	—	16
188	21	26	20	26	22	14
193	24	26	21	20	15	15
196	23	29	23	34	—	20
219	25	32	27	31	—	13
Mean	27	32	25	30	28	18

The concentrations of vitamin A in the blood at each of the first five collection periods were of the same order as those found in sheep at the Waite Institute, namely, 28γ to 38γ vitamin A per 100 ml. plasma. At the last collection period, however, they were low. This finding reflected the changes which occurred in quality and quantity of the forage during the observations which were extended over a period of progressively decreasing nutritional quality of the fodder during drought. From the termination of the favourable winter season in 1942 when 6·5 inches of rain fell between April and August, conditions became steadily worse. The total rainfall for 1943 was only 4 inches with but one effective fall of 1·75 inches in February. From then onwards until the end of the experiment the rainfall did not exceed one inch in any month. By November, 1944, the quantity of the available fodder was reduced to approximately half of that growing in October, 1942, and its quality had decreased materially. In 1942 there was good growth of spinach, geranium, various composites, spear grass and bindyi, which persisted in a dry condition into the experimental period; it had disintegrated or had been consumed by the sheep by the time the experiment had been in progress for a few months. Subsequently the flocks subsisted almost entirely on blue bush which made no fresh growth in 1943 and but little during the winter of 1944. However, although drought prevailed, the conditions were by no means severe; the quantity of feed available was adequate for the sheep to maintain their weight throughout the period of the observations.

The blood contained only traces of carotene, 3γ or less per 100 ml. serum, at each sampling period.

The concentrations of carotene in the edible portions of various fodder plants in this area are set out in Table 5.

Although the leaves of the edible trees and shrubs contained smaller concentrations of carotene than the leaves of clovers and grasses (Table 3) they were nevertheless still very good sources of carotene. The salt bushes contained a smaller concentration of carotene than the trees, the blue bushes contained still less, and bindyi contained even less carotene. Bindyi is an im-

portant fodder plant in these areas; sheep consume it in considerable quantity. When the last samples were collected the concentration of carotene in the blue bushes was rather lower than it was earlier in the experiment. The salt bushes and buckbush were then virtually absent; the bindyi was dry and contained a negligible concentration of carotene. The leaves of the edible trees were for the most part inaccessible. A combination of these factors would result in a considerable reduction of the intake of carotene by the sheep in the period preceding the final collection of blood samples.

TABLE 5.

Concentration of carotene in the edible portion of various fodder plants at Yudnapinna Station.

Species.	Carotene mg./kg. fresh material.				
	27/10/43.	9/2/44.	3/5/44.	2/8/44.	19/12/44.
Myall, *Acacia Sowdenii*	49	64	—	52	1·3*
Mulga, *Acacia aneura* (broad leaf)	—	50	59	64	68
Mulga, *Acacia aneura* (narrow leaf)	90	43	—	48	58
Cassia Sturtii	55	65	68	92	63
Bullock bush, *Heterodendon oleaefolium*	52	35	—	68	64
Buckbush, *Salsola kali*	31	25	43†	—	—
Blue bush, *Kochia sedifolia*	25	10	29	22	19
Black blue bush, *Kochia pyramidata*	21	12	17	24	11
Bladder salt bush, *Atriplex vesicarium*	43	24	33	27	—
Mallee salt bush, *Atriplex stipitatum*	62	31	43	52	—
Pop salt bush, *Atriplex spongiosum*	35	—	—	—	—
Bindyi, *Bassia obliquicuspis*	11	10	34†	14	0·10‡
Spinach, *Tetragonia eremea*	—	—	25†	19	—
Geranium, *Erodium cygnorum*	—	—	61†	61†	—
Enneapogon polyphyllus	—	—	41†	—	—
Poison weed, *Euphorbia Drummondii*	—	—	67†	—	—

* Yellowish leaves from a fallen tree.

† Young green plants 1–2 inches high.

‡ Dry plants.

The concentration of vitamin A in the livers of those of the sheep slaughtered at the Station for rations which were sampled periodically throughout the experiment varied from 420γ to 740γ per gm. liver; the concentrations of carotene in all were very low, 2·0γ to 4·5γ per gm. Although the numbers of sheep were small and the concentrations variable, the results indicated adequate reserves of vitamin A in the livers of all the sheep that were examined.

DISCUSSION.

A fluctuating intake of carotene does not appear to be reflected in the concentration of vitamin A in the blood of sheep unless the intake is low for an extended period when the concentration may fall as much as 80 p.c., Peirce (1945). In the blood of grazing sheep at the Waite Institute no significant differences were observed between the concentrations in consecutive summer and winter periods, that is between six monthly periods of very low and of high intakes of carotene. It is possible, but not likely, that the actual intake of carotene during the summer months was higher than was assessed from the analyses of gross pasture samples. Small quantities of green grass or the fallen twigs and leaves of trees, although they were of little quantitative importance may have contributed materially to the intake of carotene at this period of the year. It seems more likely that the real factor responsible for maintaining the concentration of vitamin A in the blood was the buffering effect of the large reserves in the liver. Even in young sheep with smaller reserves of vitamin A, a low intake of carotene, approximately 10γ per kg. body weight daily, failed to reduce the concentration of vitamin A in the blood until five months had elapsed, Peirce (1945). In the older sheep which had larger reserves, it is reasonable to assume that such a decline in blood concentration

would be delayed still further. Other investigators have found that the level of intake of carotene influenced the concentration of vitamin A in the blood less than it did the concentration of carotene; Davis and Madsen (1941) found that concentrations of vitamin A in the blood of cattle tended to reach a stable level, which did not increase proportionally with increasing intake of carotene; an increase in intake of carotene sufficient to bring about an eight-fold increase in blood carotene resulted in an increase of only 40 p.c. in blood vitamin A. While increases in the intake of vitamin A and of carotene were observed by Braun (1945) to bring about linear increases in the vitamin A and carotene concentrations in the blood of cattle, the former increment was less; the ratio of the two at high concentrations of carotene was only about one-fifth of that at low concentrations of carotene. This tendency is evidently even more pronounced in horses; Rasmussen, Cole and Miller (1944) were unable to detect any change in the vitamin A concentration of the blood when the intake of carotene was increased sufficiently to raise the concentration of carotene in the blood four-fold.

There is evidence that the concentration of vitamin A in the blood of sheep increases slowly with age. In the mature grazing sheep which were examined regularly for two and a half years a slight but significant increase was observed as the sheep became older. It is conceivable that this effect was due to a change in the metabolism of vitamin A. On the other hand, as will be shown in a subsequent paper (Peirce 1946), there is a progressive increase in the liver reserves with age. It is thus possible that storage becomes steadily more difficult as the amount already stored is increased.

From analyses of typical fodder plants from a station in the north-west of South Australia, on which the vegetation consisted principally of an association of myall and bluebush, it would seem that a relatively constant concentration of carotene was maintained in the available fodder for a period of nearly one year despite a very low rainfall. The concentration of vitamin A in the blood of sheep grazing there was found to be constant over this period. In the last four months, however, a number of the plants of this association disappeared and there was a decline in the carotene content of some of the others. In the final samples of blood which were collected the concentrations of vitamin A were only about two-thirds of the values recorded previously.

Large reserves of vitamin A were found in the livers of the mature sheep which were examined while grazing under normal conditions at the Waite Institute. The concentrations varied from 230γ to 810γ vitamin A per gm. liver; this was equivalent to at least 0·2 gm. vitamin A in the entire liver. Even in the two sheep examined in March and April, 1944, after they had been grazing for five months on dry pastures, the concentration of vitamin A in the liver was no lower than that found in other animals while grazing on green pastures. Sheep from the pastoral area of the north-west of South Australia were likewise observed to have large reserves of vitamin A in their livers, 420γ to 740γ per gm. These were ration sheep and so may not have had treatment comparable with the experimental animals. The concentrations of vitamin A found in the livers of grazing sheep were in the upper portion of the range found by Underwood and Conochie (1941) for sheep grazing on green pastures in Western Australia. The values which they reported were 400 I.U. to 1,000 I.U. vitamin A per gm. liver, which, if it be assumed that 1γ vitamin A is equivalent to approximately 3 I.U., become 150γ to 350γ per gm. The concentrations of 40 I.U. to 50 I.U. vitamin A per gm. liver which the Western Australian observers found in sheep which had been grazing for approximately six months on dry pasture are much lower than those of our sheep at the end of the dry period.

Carotene was present in but small amounts in all the samples of blood which were examined; the concentrations reached measurable quantities only when the intakes of carotene were high, that is when the sheep were grazing on green pasture. However, these concentrations, 5γ to 15γ per 100 ml. plasma, or 10 to 20 p.c. of the total vitamin A potency, were but a small fraction of the values that have been recorded to be present in the blood of cattle. Concentrations of 500γ to 1,000γ per 100 ml. plasma have been found to be usual in the blood of cows grazing on green pasture, Moore (1939). The concentration of carotene in the liver of the sheep was always found to be low; in general it was less than 1 p.c. of the total vitamin A potency. In cattle, the concentration of carotene in the liver is only slightly higher; carotene is stored by this animal in its adipose tissues. Analytical figures of Guilbert and Hart (1935) indicate that while only between 1 p.c. and 3 p.c. of the total vitamin A potency of the liver is present as carotene, carotene contributes over 50 p.c. to the vitamin A potency of the body fats. Sheep, as well as goats and pigs, are generally believed to convert into vitamin A the greater portion of the carotene they assimilate. This tendency is indicated by the absence of carotenoid pigments in the body fats of these animals (Palmer, 1922), and in the low concentration of carotene in their blood, which is small even when the animals have consumed, for considerable periods, fodder containing large amounts of carotene (Palmer, 1916).

SUMMARY.

In Merino sheep which grazed on natural pasture the concentration of vitamin A in the blood was found to lie between 25γ and 35γ per 100 ml. plasma. The concentration was not affected by changes in the intake of carotene from 1 mg., while grazing on dry pasture in the autumn, to 2 gm. per day while grazing on green pasture in the spring. There was, however, a slight but significant increase in the concentration of vitamin A in the blood with age. Carotene was present in the blood in measurable amounts only when the sheep were grazing on green pasture. The amounts at such times, however, were only 5γ to 15γ per 100 ml. plasma, or 10 to 20 p.c. of the total vitamin A potency of the blood.

In an area where perennial bush (*Kochia sedifolia*) formed the major constituent of the diet, the concentration of vitamin A in the blood was found to lie within the normal limits except at the end of a period of two years in which the rainfall was below normal. At this time the concentration had fallen to about two-thirds of the normal value.

In all the sheep which were examined, the reserves of vitamin A in the liver were adequate; they ranged from 230γ to 810γ per gm. liver. Carotene was present to the extent of only 2γ to 5γ per gm. liver.

Acknowledgments. My thanks are due to Mr. E. A. Cornish for the statistical examination of the experimental data, to the Waite Agricultural Research Institute for placing at my disposal its laboratory facilities and some of its sheep at Yudnapinna, to Mr. K. Woodroffe, under whose direction the grazing trial at Yudnapinna was being conducted, for valuable advice, and to Mr. A. W. Dick, overseer at Yudnapinna, for his assistance in the collection of the blood, liver and fodder samples. I should also like to acknowledge the financial support provided by the Australian Wool Board through its grants to C.S.I.R. Finally, I wish to thank Mr. H. R. Marston, Chief of the Division of Biochemistry and General Nutrition, for his advice during the experiment and for his helpful criticism during the preparation and correction of the manuscript.

REFERENCES.

Braun, W. (1945): J. Nutrition, 29, p. 61.
Crocker, R. L. and Skewes, H. R. (1941): Trans. Roy. Soc. S. Austral., 65, p. 44.
Davis, R. E. and Madsen, L. L. (1941): J. Nutrition, 21, p. 135.
Guilbert, H. R. and Hart, G. H. (1935): *Ibid.*, 10, p. 409.
Kemmerer, A. R., Fudge, J. F. and Fraps, G. S. (1942): J. Amer. Soc. Agronom., 34, p. 851.
Moon, F. E. (1939): Emp. J. exp. Agr., 7, p. 225.
Moore, L A. (1939): J. dairy Sci., 22, p. 513.
Myburgh, S. J. (1935): Onderstepoort J. vet. Sci., 5, p. 475.
Myburgh, S. J. (1940): *Ibid.*, 14, p. 421.
Palmer, L. S. (1916): J. biol. Chem., 27, p. 27.
Palmer, L S. (1922): Carotenoids and related pigments. Amer. Chem. Soc. Monograph.
Peirce, A. W. (1945): Austral. J. exp. Biol., 23, p. 295.
Peirce, A. W. (1946): In press.
Peterson, W. J. (1941): Ind. eng. Chem., Analyt. Ed., 13, p. 212.
Rasmussen, R. A., Cole, C. L. and Miller, M. (1944): J. animal Sci., 3, p. 346.
Snyder, W. W. and Moore, L. A. (1940): J. dairy Sci., 23, p. 363.
Underwood, E. J. and Conochie, J. (1941): Austral, vet. J., 17, p. 202.
Underwood, E. J. and Conochie, J. (1943): *Ibid.*, 19, p. 37.
Underwood, E. J. and Curnow, D. H. (1944): *Ibid.*, 20, p. 248.
Wall, M. E. and Kelley, E. G. (1943): Ind. eng. Chem., Analyt. Ed., 15, p. 18.
Woodroffe, K. (1941): J. Austral. Inst. agric Sci., 7, p. 117.

MORPHOLOGICAL, INHERITANCE AND GROWTH STUDIES OF THE K SALTATION PRODUCED SELECTIVELY BY SHORT WAVELENGTHS OF ULTRA-VIOLET IRRADIATION IN THE FUNGUS *CHAETOMIUM GLOBOSUM* KUNZE (ASCOMYCETES, SPHAERIALES)

by JOAN MUNRO FORD

(From the Physics Department, University of Tasmania).

(Accepted for publication 3rd June, 1946.)

In a previous paper, McAulay, Plomley and Ford (1945), attention was drawn to a type of change produced selectively in large numbers of fungus colonies grown from spores irradiated at short wavelengths of ultra-violet light. The modification was designated by the letter "K", and was thought to be unstable in behaviour in the sense that a colony derived from the treated spore might have two distinct forms. As this modification was produced selectively it has been considered of interest to examine it in more detail, and this paper describes features of its morphology, inheritance and growth. The K modification has been obtained in colonies grown from irradiated spores of two Chaetomium species, *Chaetomium globosum* and *Chaetomium elatum*, but the latter species has not been studied in detail.

Further experiments have shown that the "K" is more clearly defined in behaviour and characteristics than at first was thought. This has been increasingly manifested in later work, and it is considered justifiable to call it a saltation. The term saltation has been used to cover K mycelial saltants and K spore saltants. A detailed study of inheritance in 80 colonies showed that the characteristics of "K" could always be produced from mycelial inoculations, and two colonies also produced them from single spore inoculations. All K saltations therefore are mycelial saltants and two of them are also spore saltants.

The morphology of the K saltants was almost identical, whether reproducible through spores or not. The growth in diameter of K saltations was peculiar to all K colonies. Growth was periodic and ceased when a colony diameter of approximately 50 mm. was attained, but continued again after a few days.

MORPHOLOGICAL STUDIES.

The Normal Colony.

The parent strains used in the irradiation experiments were *Chaetomium globosum* Lj, Kb and a *globosum* saltant Fld, McAuley, Plomley and Ford (1945). The present studies have been confined mainly to Fld, a saltant produced in irradiation experiments.

The normal colony of Fld (Fig. 1a and 1b) when grown in 9 cm. Petrie dishes on 2 p.c. malt agar, which was the medium used throughout the experiments, has certain standard features. The aerial mycelium is fawn coloured, not dense, with perithecia yellow in colour, numerous, 100μ–300μ in length, situated on surface and slightly sub-surface hyphae and distributed evenly over the colony. The cellular wall of the perithecium is 2 or 3 cells thick, and lateral remotely-septate hairs grow from some of these cells. When mature, spore protrusion is abundant and few terminal hairs at present. The asci are 8 spored and spore dimensions are 10μ × 8μ and those examined appeared to be uninucleate. The dark colour and dense contents of the mature spores made nuclear observations uncertain. Viable spores form 3 weeks after first plating of the original single spore which grows into a new colony.

K Saltation.

Irradiation of the spores of *Chaetomium globosum* at the short wavelengths 265 mμ and 280 mμ produced the K saltation in an average of 30 p.c. of the colonies grown from spores surviving irradiation.

The K saltation (Fig. 2a, 2b and 2c) shows certain constant features whether it forms a small sector or the whole colony, and whether it grows from irradiated spores or spore and mycelial inoculations from a K colony. Growth in diameter is arrested at the growing edge of the colony and the first sign of a "K" area or sector is the appearance of a small dent at the colony edge. The aerial mycelium is the first to stop growing and the sub-surface mycelium is the last. Hyphae are sparse at this stage and some of the hyphae possess degenerate cytoplasm. A few days after cessation of growth at the margin some hyphae grow to the surface and at isolated points develop into flares of surface and aerial mycelium (Fig. 3). When the K colonies are grown in 9 cm. diameter Petrie dishes these late forming flares may appear normal near the side of the Petrie dish or they may very soon develop the characteristic K arrested margins (see Fig. 2c). If allowed to grow in 20 cm. Petrie dishes, where they do not meet an obstruction so soon, the normal-looking flares often develop the characteristic K arrested edge and brown pigmentation.

Small sectors of "K" have few or no perithecia but show other characteristic K features such as the arrested edge (Fig. 2a) and sometimes brown pigment and late forming flares, the last two depending more on the size of the sector than the first. Inside the arrested edge of the large K sectors brown pigment develops and white knots of mycelium (Fig. 3), which later develop numerous perithecia (Fig. 2c), form outside the zone of pigment. The sectors, both small and large, are sharply separated from the adjacent normal by the abrupt cessation of the numerous normal perithecia. As many as 6 K sectors alternating with normal sectors have been observed in one colony grown from an irradiated spore. When the "K" forms large sectors or the whole colony (Fig. 2c) a marked zone of brown pigment develops in the surface hyphae, and particularly the upper sub-surface hyphae, has a width of approximately 1 cm. in the widest part and is 1 cm. inside the arrested edge of the colony (Fig. 2c). The pigmented region forms shortly after the arrested edge becomes apparent. Hyphae in the brown zone of the K saltation have yellow-brown to dark brown cells at intervals. Sometimes a brown cell occupied a central position, sometimes a terminal position and sometimes appeared at the junction of two hyphal branches. When the pigment was dark brown the coloured cells looked like plugs between the fairly normal cells either side of it. The pigment usually lined the walls of the cell but sometimes appeared as small to large highly refractive granules spread throughout the cell. It was not soluble in the ordinary solvents tried: water, ether, alcohol and xylol. Nothing like these brown cells was observed in the normal colonies. When examined under 1,000 times magnification some of the cells in the brown zone were irregular in outline with densely granular contents and nuclei varying in size from small fragments to larger than normal. The hyphal branches in the brown zone and the sparser zone further out were often stunted and few in number.

On the outer side of the brown zone conspicuous white knots (Fig. 3a and 3b) of surface and aerial mycelium develop at the same time as perithecia are forming in the centre and normal sectors of the colony. These white knots were observed to be composed of massed hyphae of all sizes, fine, medium and wide, and compared with normal hyphae (Fig. 4a), the last especially were chain-like and nobbly in structure (Fig. 4b). These hyphae took part in the development of the late forming perithecia. The mycelium becomes fairly dense in this region and the perithecia, although late forming, are abundant.

The perithecia in the zone with little aerial mycelium just beyond the arrested edge, usually remain small and immature.

INHERITANCE OF THE K SALTATION.

Two methods used to test out the way in which the K saltation could be reproduced, firstly mycelial inoculations and secondly single spore inoculations, showed that the saltations could always be obtained by the first method and occasionally also by the second method.

EXPLANATION OF FIGURES.

Fig. 1. *Chaetomium globosum.* Normal colonies of Fld, a saltant which turned up in irradiation experiments. 1a, reflected light; 1b, transmitted light. × ¾.

Fig. 2. K saltations. Colonies grown from spores irradiated at short wavelengths of ultra-violet light. 2a. 2 small K sectors adjacent to normal sectors. 2b. 4 K sectors and 4 normal sectors. 2c. Total K saltation colony. The colony has a centre with numerous perithecia surrounded by a zone with few perithecia and with brown pigment (A) in the surface and upper sub-surface mycelium of most of this zone. Surrounding the brown pigmented zone is a zone with numerous perithecia (B) which have developed in the region of white knots of mycelium which were conspicuous soon after this region was arrested in its growth (see above and Fig. 3). The outer edge of the perithecia in this zone marks the original arrested edge of the colony. Aerial mycelium and perithecia are sparse in the next zone (C) which marks the first part of the late forming flares (see above). These flares may soon develop characteristic "K" features such as arrested growth of the edge (D) or may appear comparatively normal (E) until the Petrie dish edge obstructs further growth. 2a, × ¾; 2b and 2c, × 1.

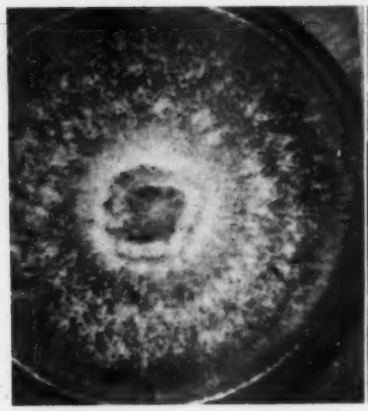


Fig. 1a.

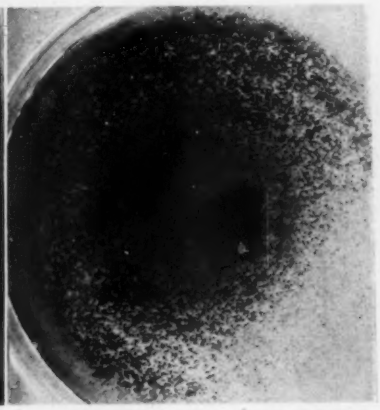


Fig. 1b.

Fig. 2a.

Fig. 2b.

Fig. 2c.

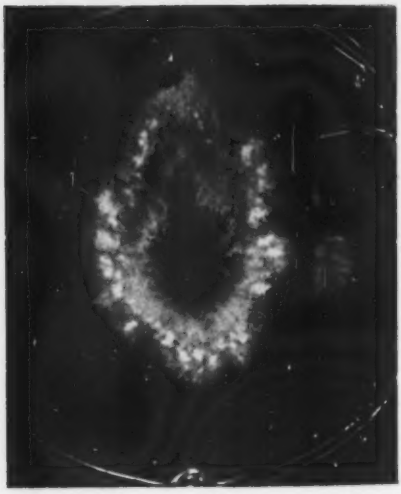


Fig. 3a.

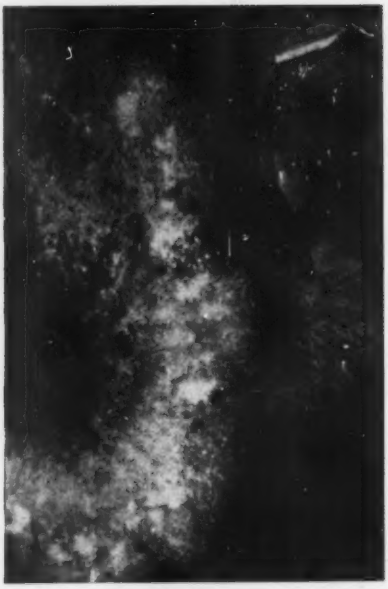


Fig. 3b.

Fig. 3. 3a. Total K saltation colony showing white knots of hyphae on outer side of the brown pigmented zone. × ¾. 3b. A portion of 3a magnified, × 1½. An isolated flare of new growth beyond the arrested edge is apparent on the right of the colony.

K mycelial saltants. In the first method small pieces of mycelium were taken from various parts of the colonies showing K characteristics and plated in separate Petrie dishes of 2 p.c. malt agar. About 80 colonies showing the K saltation features and taken from 20 different short wave-length experiments were sub-cultured in this way. It was found that certain regions of the colonies were more likely to reproduce the K characters than others. These parts were just inside the brown pigmented zone, the brown zone itself, and when the K sector was small the mycelium anywhere along the arrested border. In the great majority of cases mycelium taken from these areas produced a total K saltation colony or a colony composed of K sectors and normal sectors. This was so through 4 generations of mycelial inoculations. Mycelial inoculations taken from other regions of the K sectors or colonies sometimes produced the K features and sometimes produced the normal. Inoculations taken from the normal sectors usually developed into total normal colonies.

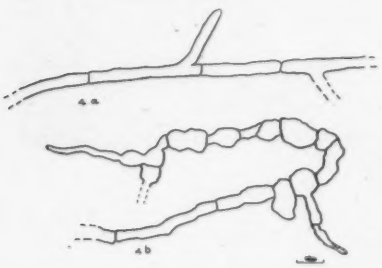


Fig. 4. 4a. Diagram of portion of a normal hypha. 4b. Diagram of portion of a hypha from a white knot: a wide hypha showing nobby appearance of the cells and stunted branches. × about 1,200.

Immature perithecia were grown in separate Petrie dishes and several formed the K saltation.

Reproduction from single spores. The second method of studying inheritance was that of single spore inoculations. Spores were plated singly, with a pointed needle on clear malt agar. With a magnification of 60 times they appeared as big as pins' heads, and with a little practice could be quickly picked up and placed at marked intervals on the clear medium. It is important to stress that in all cases spores were plated singly so that there was no possibility of 2 types of mycelium being mixed together. The single spores were examined each day for germination and when the colonies were visible to the naked eye they were transferred to separate dishes of malt agar and further development watched.

TABLE 1.

Showing the number of germinating single spores and the number of those forming "visible" colonies. Spores were taken from normal colonies and K saltations of the two kinds, (1) forming K through spore sub-cultures and (2) not forming K through spore sub-cultures.

Colonies from which single spores taken.	No. of single spores plated.	No. of single spores germinating.	No. of germinated s/s forming "visible" colonies.	No. growing to form K saltations.
Normal control colonies	500	440	440	0
K saltations				
1. Ka and Kb through 7 and 2 generations respectively	320	284	227	227*
2. 65 K saltations through 2 generations	763	648	644	0

* Although a number of these colonies resulting from single spore inoculations did appear at first to be normal they possessed the same early slow rate of growth as the other Ka and Kb spore colonies (Fig. 5), and if grown on large Petrie dishes produced the characteristic K arrested edge.

Single spore inoculations totalling approximately 1,080 (see Table 1) were made from 67 K saltation colonies taken from 12 different short wavelength experiments. Sixty-five of the colonies produced only normal colonies on single spore sub-culture but 2 colonies produced the K characteristics. These 2 saltants have been called Ka and Kb for convenience and their early peculiarities of growth are described in the next section. The Ka (Fig. 5) and Kb saltants

Fig. 5a.

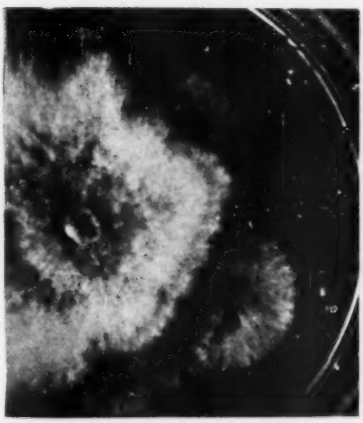


Fig. 5b.

Fig. 5. 5a. A K spore saltant colony showing a total K effect and grown from a third generation spore sub-culture. Transmitted light × ¾. 5b. A K spore saltant colony grown from a mycelium inoculation. Reflected light. × 1.

have produced K characteristics through 7 generations and 2 generations respectively of single spore sub-cultures from all parts of the colonies. The 65 other K saltations have produced only normal characteristics through 2 or 3 generations of single spore inoculations. Spores were taken from all parts of the K colonies, from normal sectors and K sectors, and from total K colonies. Spores taken from any part of the Ka and Kb spore saltant colonies produced the K characteristics, whereas the spores taken from any part of the K mycelial saltants reproduced normal features only.

On mycelial sub-culture (Fig. 5b) the Ka and Kb saltants formed colonies similar to those produced by single spore inoculations (Fig. 5a). Colonies grown from spore and mycelial inoculations of the Ka and Kb were almost identical with the other K saltations. The K spore saltant colony in Fig. 5a can be distinguished from the normal colony (Fig. 1b) by the zone of

strong brown pigment and rather dense perithecia developed inside the region of the original arrested edge. (See also Fig. 5b.) Late-forming flares which are similar to those marked "D" in Fig. 2e, a total K mycelial saltant, are also present in Fig. 5.

GROWTH STUDIES.

K Saltations and Comparison with Normal.

The average diameter of the fungal colonies at intervals of several hours was taken as a measure of increase in growth with time. The average diameter of the colony was obtained by measuring two directions at right angles to each other and calculating the average from these. When small, the colonies grown from single spore inoculations were measured with eyepiece micrometers, and when larger with a millimeter rule. Examination of the the colonies was made from the time of spore plating and the spore (average diameter 9μ) has been included in the measurements.

The number of spores germinating and growing to form "visible" colonies in normal *globosum* and spore inoculations from K saltations has been listed in Table 1. Germination was said to have occurred when the germ tube or first hypha was more than twice the spore length. Germinating spores were said to have formed "visible" colonies when they were visible to the naked eye (at about 1 mm. diameter).

The normal colonies of *Chaetomium globosum* form spores which when ripe germinate 4 to 8 hours after spore plating. The germinated spores normally form "visible" colonies about 24 hours after germination.

It will be observed from Table 1 that the percentage of Ka and Kb germinating spores which formed "visible" colonies was 80 p.c. (227/284), a lower figure than that for "visible" colonies from spore inoculations of the K saltations other than Ka and Kb which was 99 p.c. (644/648), and the normal which was 100 p.c. (440/440).

In the K spore saltants germination of the spores usually took place normally, i.e. at about 6 hours after spore plating, but in forming "visible" colonies there was a delay which varied from 3 to 15 days.

TABLE 2.

Showing percentage of germinating spores which formed "visible" colonies, and percentage of K saltations in colonies grown from spores surviving irradiation in experiments at 265 mμ. Two strains of Chaetomium globosum *have been tabulated (Lj and Fld, a* globosum *saltant). Spores were taken from colonies varying in age from 1½ months to 4 months.*

Colony, age and strain.	Experiments at 265 mμ. Percentage of germinating spores forming "visible" colonies.	Percentage of K saltations in "visible" colonies.
1½ months Fld	6	39
	27	16
	78	9
	83	4
2 months Lj	2	44
	16	41
	68	14
3 months Fld	25	50
	69	37
4 months Lj	46	40
	60	27
	74	24

When the spores of *Chaetomium globosum* were irradiated by the short wavelengths of monochromatic ultra-violet light, germination was sometimes delayed from 1 to 4 days. Many colonies did not become "visible", the number becoming "visible" depending on the dose of irradiation. The greater the dose the greater was the number of colonies that did not attain the "visible" stage. Colonies grown from irradiated spores produced the K saltation in about 30 p.c. of the "visible" colonies at the short wavelengths. Table 2 shows the percentage of "visible" colonies and the percentage of K saltations in experiments at the short wavelength of ultra-violet, 265 mμ. Only experiments with more than 30 experimental colonies were chosen for analysis of saltations. No K saltations occurred in the normal control colonies.

The average diameter of colonies of normal and K saltations has been plotted against time in Figs. 6, 7 and 8. In Fig. 6 the average diameter is plotted as the logarithm of diameter

in mm. and the early growth rate only is recorded. In Fig. 7 the average actual diameter in mm. of later growth of colonies is plotted. The graph of logarithmic growth gives examples of colonies grown from single spores and the next graph gives examples of colonies grown from single spores (full dots), and also from mycelial inoculations (hollow dots). The spore has been included in the measurements of the colonies grown from single spores. In the case of mycelial sub-cultures the inoculation was fairly large, being about 5 or 8 mm. in average diameter. A period of about one day was necessary for the mycelial inoculation to acquire a steady rate of growth. In Figs. 6 and 7 the normal colonies (A) are represented by unbroken lines, the K spore saltant colonies (C) by broken lines, and the K saltations (other than the spore saltants) and the "non-visible" colonies from irradiated spores (B) are represented by dotted lines. Fig. 8 is a diagrammatic representation of growth in diameter of normal and K saltation colonies.

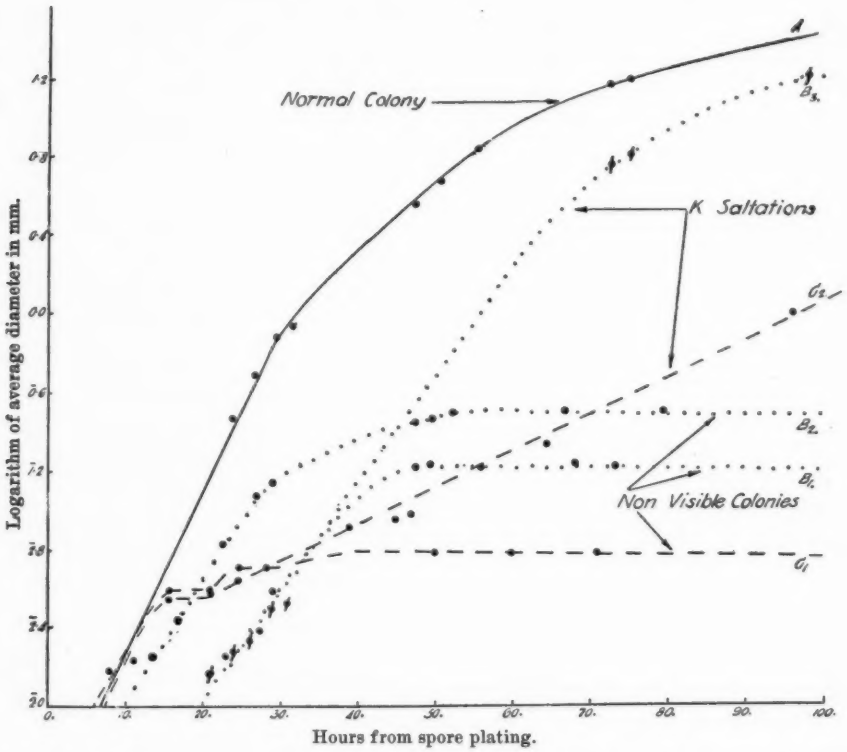


Fig. 6. Showing logarithm of average diameter in mm. of early growth of colonies plotted against time in hours from plating of the spores. The continuous line A represents a normal colony. The dotted lines B represent colonies grown from irradiated spores; B_1 and B_2 are "non-visible" colonies and B_3 is a K saltation. The broken lines (C) represent colonies grown from K spore saltant colony spores, C_1 is a "non-visible" colony and C_2 is a Ka spore saltant.

The normal colony (A) has a steady logarithmic growth from the time of germination, here at 7 hours after spore plating, until about 20 hours later (Fig. 6) when the logarithmic rate changes. At about 50 hours after spore plating and at a diameter of 6 mm. (see average actual diameter Fig. 7), growth rate becomes constant. This constant rate is maintained until the edge of the Petrie dish is reached (Plomley and Ford, unpublished). The colony grown from a mycelium inoculation (A_2) also shows a constant rate of growth from about 50 hours after plating of the mycelium inoculation until the Petrie dish edge is reached.

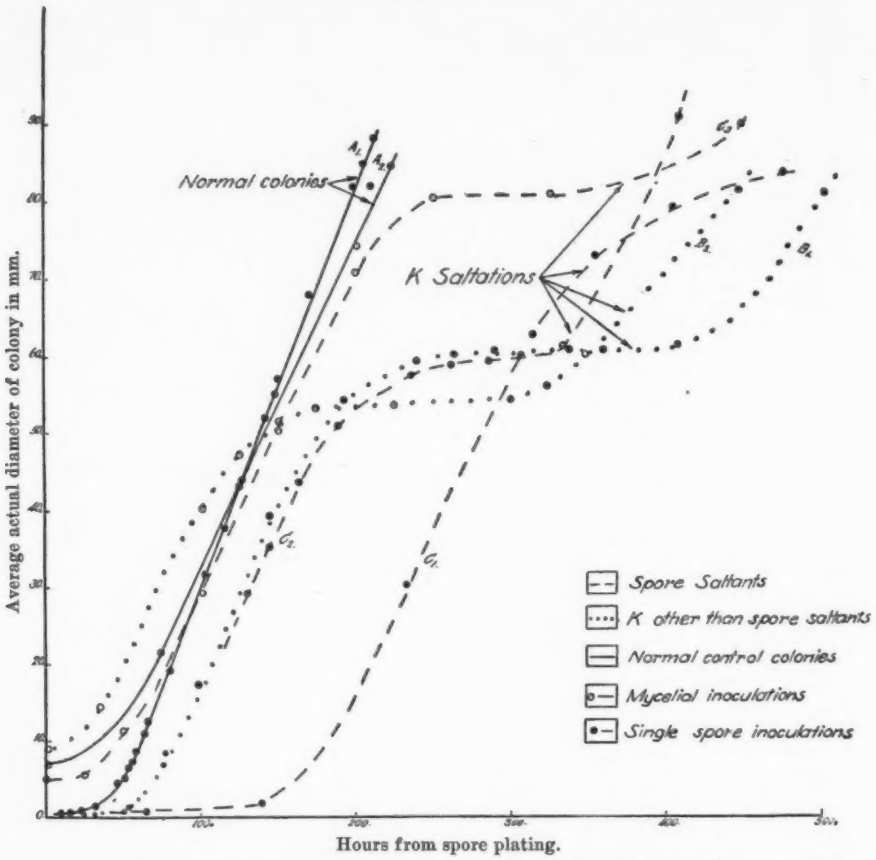


Fig. 7. Showing average actual diameter in mm. of later growth of colonies plotted against time in hours. The large solid dots represent colonies grown from single spores and the hollow dots represent colonies grown from mycelial inoculations. The continuous lines (A) are normal colonies, the dotted lines (B), K saltations other than the K spore saltants, and the broken lines (C) are K spore saltant colonies; C_1 and C_2 are K spore saltants grown from single spores and C_3 is a K spore saltant grown from a mycelial inoculation.

Colonies grown from irradiated spores and plotted logarithmically in Fig. 6 are represented by the dotted lines B_1, B_2 and B_3. The time of germination was delayed in the colonies grown from irradiated spores and it will be seen from the graph that one colony (B_2) germinated 10 hours after spore plating and that two colonies (B_1 and B_3) germinated 20 hours after spore plating. Two colonies (B_1 and B_2) ceased growing altogether about 50 hours after spore plating and one colony (B_3) became a K saltation. The first two did not resume growth and were "non-visible" colonies. The colony which became a K saltation (B_3) showed 40 hours of slow logarithmic growth and a uniform rate (see also Fig. 7, B_1) 70 hours after spore plating, and this was maintained until a colony diameter of about 50 mm. was attained. Growth then slowed down and ceased altogether for 4 or 5 days. After this period growth recommenced and 50 hours later a constant rate was resumed which was similar to that of the previous period of constant growth. The second constant rate is usually maintained until the edge of the Petrie dish is reached. If new K flares appear at the margin of the colony cessation of growth may again occur. If allowed to continue indefinitely on unlimited medium the K saltation could probably produce a growth rate showing periods of no growth alternating with periods of constant

growth. The mycelial inoculation from a K saltation colony (Fig. 7, B_2) also showed the constant trend of growth beginning at about 50 hours after plating the inoculation, and cessation of growth which occurred about 170 hours after plating. A constant growth rate here also is resumed after this period of cessation of growth.

The growth rate of the two K spore saltants (C, broken lines) is shown by examples in the two graphs, the logarithmic early growth (Fig. 6) and the actual later growth (Fig. 7). In Fig. 6 the growth rates of two colonies (C_1 and C_2) grown from single spores of a Ka saltant colony have been plotted. Germination occurred normally at about 7 hours after spore plating. A marked change occurred after about 7 hours' growth, i.e. 14 hours after spore plating, evident in the much slower rate. One colony (C_1) ceased to grow at about 40 hours after spore plating (a "non-visible" colony). The other colony (Fig. 6, C_2), grown from a Ka single spore, showed a slow logarithmic rate which was acquired and maintained for about 100 hours (see also later growth of the same colony, C_1 in Fig. 7). There was then a change and about 50 hours later a constant rate was acquired and maintained until the colony diameter reached 65 mm. approximately. Growth was then very slow in characteristic K fashion until the Petrie dish edge was reached. As growth did not cease altogether this may have been a colony in which cessation occurred very near the edge of the Petrie dish. Lack of absolute cessation of growth may also have been due to certain portions of the colony continuing to grow while other parts had ceased.

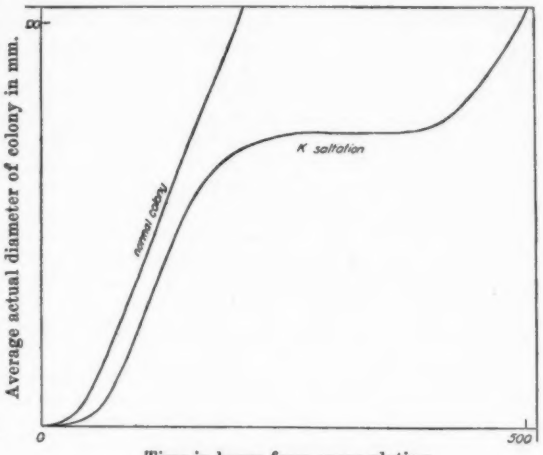


Fig. 8. Comparative growth of a K saltation and a normal colony.

The growth rate of a Kb colony (C_2) grown from a single spore is plotted in Fig. 7. Growth was not measured for the first 30 hours after plating. Typical K features of growth were present in the later growth. The mycelial inoculation from a Ka colony (Fig. 7, C_3) showed the same growth trend as the mycelial inoculation from the K saltation other than Ka and Kb. Cessation of growth occurred at about a colony diameter of 80 mm. for a period of 3 days approximately, and after that period growth was again resumed at a constant rate.

DISCUSSION.

The K character is an instability produced selectively by short wavelengths of ultra-violet light in colonies grown from irradiated spores of Chaetomium. It is known to occur in *Chaetomium globosum* and *Chaetomium elatum*. It is an instability in that the hyphal cells of the mycelium may form either normal or K characteristics. The K instability can always be reproduced from certain parts of the mycelium of K colonies and in two cases[1] it has been reproduced through several generations of spore sub-cultures. It is thought justifiable to call the "K" a saltation, not so much because of the "K's" which have been reproduced through spores, but on account of the constant characteristics of the plant which make recognition of the type very certain. The type can always be reproduced through mycelial transplants, but the "K" is not a mere vegetative change, as the K characteristics have been obtained through single spore reproductions in several cases. One of the latter has been produced through seven generations sub-cultured

[1] Since submitting this paper for publication three more K colonies have been found to reproduce the K characteristics through single spore inoculations, i.e. three more K spore saltants have been discovered.

over a period of about two years. It is convenient to describe those K colonies which reproduce the K features through mycelial inoculations only, as mycelial saltants, and to describe those which reproduce the features through spores as well as mycelial inoculations as spore saltants.

In short wavelength experiments the K saltation is produced in about 30 p.c. of all the experimental colonies grown from irradiated spores (Table 2). In one experiment 50 p.c. of the experimental colonies were K saltations. The total percentage of all other saltations in experimental colonies at the short wavelengths of ultra-violet light tested totalled 4 p.c. (McAulay, Plomley and Ford, 1945).

There is some variation in the way in which the K saltation can be reproduced. Spore inoculations from 67 K colonies grown from irradiated spores showed that two colonies always reproduced the "K" through spores. The other 65 saltations did not reproduce the "K" through spores, the resulting colonies always being normal. The ability to reproduce the "K" through spores in these colonies must have been lost during the actual formation of the spores in the perithecia. It was found that immature perithecia transplanted before ripening of the spores would sometimes grow to form K saltations. As the spores could not produce them, the K characters must have been potentially present in the cellular wall of the perithecium, and the potentiality to form "K" must have been lost in actual spore formation.

The K saltation is characterized by certain peculiarities of growth which always occur in the "K" whether it is grown from a mycelial sub-culture, irradiated spore or spore sub-culture. The diagrammatic Fig. 8 shows the differences between growth in average diameter of normal and K saltation colonies. Growth ceases for a time when the K saltation colony or sector has reached an average diameter of between 50 mm. and 80 mm. This would suggest that staling might have occurred, but, after a period of no growth for three to ten days, growth is renewed at the margin. This feature is characteristic of all K saltations (Fig. 7). In the K saltations the effect of the irradiation on the spores is delayed and the characteristics associated with the effect have been reproducible through many generations.

SUMMARY.

Peculiarities of the morphology, inheritance and growth of an instability which is produced selectively by short wavelengths of ultra-violet light have been described.

The K instability can always be reproduced from certain parts of mycelium and in a number of cases it has been reproduced through several generations of spore sub-cultures.

It is now thought justifiable to call this instability a saltant change.

When a diameter of between 50 mm. and 80 mm. is reached, growth of the colony or the sector is periodic and ceases for three to ten days but is renewed again after this interval.

The saltation is produced on an average in 30 p.c. of colonies surviving irradiation at the short wavelength 265 mμ. In one experiment there were 50 p.c. of the K saltation colonies.

The K saltation has not appeared in control colonies, and is almost absent with long wavelengths of monochromatic ultra-violet irradiation.

Acknowledgments. Sincere thanks are expressed to Professor A. L. McAulay for many helpful suggestions and criticisms during the course of this work. The funds for the work came from the Commonwealth Research Grant to the University.

REFERENCES.

McAulay, A. L., Plomley, N. J. B. and Ford, J. M. (1945): Austral. J. exp. Biol., 23, p. 53

Plomley, N. J. B. and Ford, J. M. (unpublished work): Colony formation in the fungus *Chaetomium globosum* Kunze.

A NOTE ON THE EFFECT OF NICOTINIC ACID ON THE GROWTH OF THE TESTES AND SEMINAL VESICLES IN IMMATURE MALE RATS

by ROSS WILSON HAWKER

(From the Royal Hobart Hospital, Tasmania).

(Accepted for publication 29th May, 1946.)

In a recent experiment (Hawker, 1946) it was shown that nicotinic acid stimulated the rapid proliferation and maturation of Graafian follicles in the ovary of the juvenile virgin guinea-pig, and caused an almost proportionate decrease in the weight of the pituitary gland.

With knowledge of this evidence, the following experiment was conducted to see whether nicotinic acid has any effect on the male generative organs.

Ten male rats of Wistar strain, 36–40 days old when killed, were used: five controls and five experimentals. They were fed milk, bran, bread, fresh green grass and cabbage leaves. The experimental animals were given daily injections of 25 mg. of sodium nicotinate for the ten days prior to killing.

The animals were chloroformed and the testes and seminal vesicles removed, dried, weighed and placed in formalin. The results are shown in Table 1.

TABLE 1.

Experimental animals. No.	Weight in gm. (both testes).	Weight in milligrams (both seminal vesicles).
1	2·025	34·0
2	1·750	19·0
3	1·840	39·7
4	2·425	27·3
5	2·150	15·2
	Average = 2·038 gm.	Average = 27·2 mg.
Controls. No.	**Weight in gm. (both testes).**	**Weight in milligrams (both seminal vesicles).**
1	2·470	53·5
2	2·780	94·0
3	2·660	76·0
4	2·270	26·0
5	2·310	54·3
	Average = 2·498 gm.	Average = 60·7 mg.

Statistical analyses: Testes, $t = 2{\cdot}97$; for $n = 8$, $\cdot02 > P > \cdot01$.
Seminal vesicles, $t = 2{\cdot}71$; for $n = 8$, $\cdot05 > P > \cdot02$.

DISCUSSION.

It is well established experimentally that injections of follicular hormone and castration will effect a loss of weight in the male generative organs.

Price and Ortiz (1944) found that daily injections of estradiol benzoate (I.R.U.) for six days into rats, killed at 36 days, caused a decrease of 9 p.c. and 22 p.c. respectively in the weight of the testes and seminal vesicles. The ovaries of their treated females were 40 p.c. heavier than control ovaries, and this increase was due to follicular proliferation.

Hays and Mathieson (1945) have shown that in 50-day-old rats castrated at 20 days, the mean seminal vesicle weight was 18·1 mg. compared to 44·3 mg. in the controls.

A decrease of 21·3 p.c. testicular weight in four-week-old rats receiving testosterone propionate (1 mg. per kg. body weight) is reported by Steadman and Krichesky (1945).

It would thus appear that nicotinic acid has an estrogenic effect in the juvenile male rat. Possibly this effect is mediated through inhibition of the anterior pituitary in its production of gonadotropins, thus removing from the male generative organs their normal androgenic stimulation.

SUMMARY.

The daily injection of sodium nicotinate (25 mg.) into immature male rats resulted in a significant decrease in the weights of the testes and seminal vesicles.

Acknowledgments. Many thanks to Sister Worthington, who assisted with the experiment, and also to Dr. Campbell Duncan and Mr. R. J. Ralph.

REFERENCES.

Hawker, R. W. (1946): Med. J. Austral., 1, p. 872.
Price, D. and Ortiz, E. (1944): Endocrinology, 34, p. 215.
Hays, H. W. and Mathieson, D. R. (1945): *Ibid.*, 37, p. 266.
Steadman, F. H. and Krichesky, B. (1945): *Ibid.*, 37, p. 89.